Genetic risk factors for venous thrombosis:

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Genetic risk factors for venous thrombosis: key players or minor risk modifiers?

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Introduction

Blood coagulation

The arrest of bleeding (hemostasis) after an injury is the result of interplay between the damaged blood vessel, circulating cells (thrombocytes) in the blood, and the blood coagulation system. Ultimately, a hemostatic plug is formed, which halts bleeding during the repair process of the disrupted vessel. In the aftermath, feedback processes will control extension and initiate lysis of the blood clot. A central factor in the blood coagulation process is thrombin (factor IIa), which is formed from prothrombin after a series of enzymatic reactions. Thrombin generation is initiated when small amounts of factor VIIa bind to tissue factor (TF), which ordinarily will only be exposed to flowing blood after damage to the endothelium (Figure 1).[1] The factor VIIa-TF complex activates factors IX and X, which with the help of the activated cofactors VIII and V will convert prothrombin to thrombin (Figure 1). Thrombin generation is inhibited by tissue factor pathway inhibitor (TFPI), antithrombin and activated protein C (APC). TFPI blocks the tissue factor-initiated activation of factors IX and X, and antithrombin inactivates factor IX, factor X and prothrombin. APC is formed when thrombin binds to thrombomodulin, a receptor on the endothelium, and inactivates cofactors V and VIII. Defects in the hemostatic balance maintained by the pro- and anticoagulant systems can produce hemorrhagic or thrombotic disease.

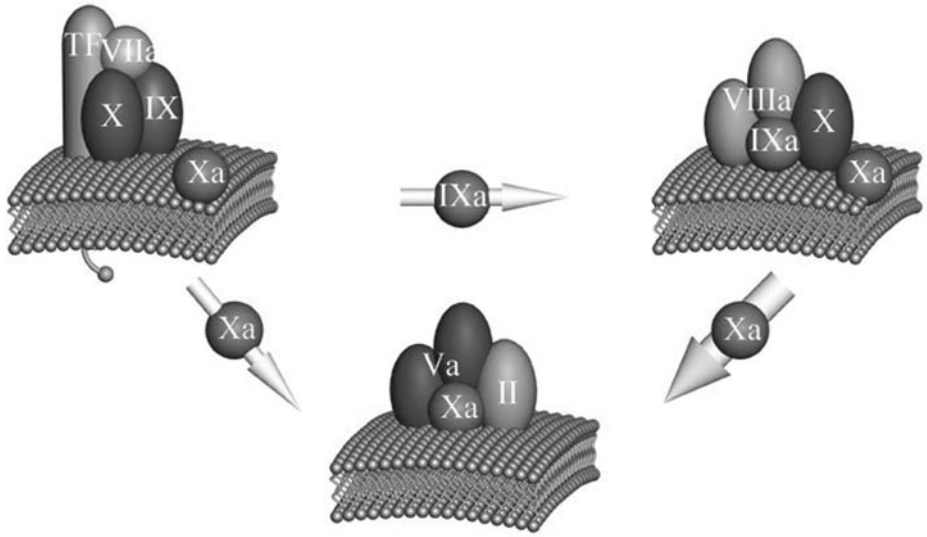

Figure 1 *Derived with permission from KG Mann. Biochemistry and Physiology of Blood Coagulation. Thrombosis and Haemostasis 1999;82:165-174.*

Venous thrombosis

Venous thrombosis affects 1-2 per 1000 individuals per year.[2,3] The most frequent clinical manifestation is thrombosis in the deep veins of the leg, which may embolize to the lungs. Major consequences can be chronic leg or lung problems, which may have a major impact on quality of life[4,5], and may cause death.[6] Numerous risk factors are known to increase the risk of venous thrombosis such as hereditary defects in the coagulation system, hormonal changes (e.g., pregnancy, use of female hormones), other diseases (e.g., malignancy), and physical factors (e.g., inactivity).[7] One or more risk factors can now be found in one-third of consecutive patients with a first deep venous thrombosis.[8] Also, one-fourth of all consecutive patients with a first event report at least one first-degree relative with venous thrombosis.[9]
The first identified hereditary defect in the coagulation system increasing the risk of venous thrombosis was antithrombin deficiency[10], which was followed by the discovery of deficiencies in other natural coagulation inhibitors: protein C[11] and protein S[12]. The most common hereditary defects were discovered within the last decade: factor V Leiden[13] and the prothrombin G20210A variant[14]. Although testing for these defects is common, a positive outcome does not always predict or fully explain disease occurrence due to the low penetrance of disease associated with these defects. Like many other common diseases, venous thrombosis is believed to be a multicausal disease, in which one risk factor is seldom sufficient to cause venous disease.[15] For example, Miletich *et al*. reported in 1987 that protein C deficiency was quite common in the normal population without a detectable association with a risk of thrombosis[16], whereas in family studies a strong association was found.[17,18] This discrepancy can be explained by the multicausal nature of the disease: multiple defects are present within families leading to a familial tendency for venous thrombosis (thrombophilia), as shown by Lensen *et al*.[19,20] Familial thrombophilia is characterized by a strong family history of venous thrombosis with an early age of onset, recurrent events, severe events out of proportion to known stimuli, and unusual clinical presentation of the disease.[21]

Outline of this thesis

This thesis focuses on the risk of venous thrombosis associated with familial thrombophilia, and on the search for new genetic risk factors. The results of two studies will be described: the European Prospective Cohort on Thrombophilia (EPCOT) study and the International Protein C Investigation (IPCI).

The European Prospective Cohort on Thrombophilia (EPCOT)

As large studies on the risk of venous thrombosis in families with inherited thrombophilia were scarce in the 1990s, whereas the demand for therapeutic guidelines was high, it was decided to initiate a European prospective cohort study on thrombophilia to obtain reliable risk estimates for first and recurrent events. Whereas the primary aim was to estimate the risk of thrombosis, secondary aims were to investigate other effects of thrombophilia, such as fetal loss, as well as the risk of side effects of treatment, such as hemorrhage following anticoagulant therapy. A total of nine thrombosis centers (from Barcelona, Bologna, Frankfurt, Glasgow, Leiden, Malmö, Paris, Sheffield and Vienna) with long-standing interest in thrombophilia research participated in the European Prospective Cohort on Thrombophilia, which was funded by the European Union as a Concerted Action. Patients referred to these specialist clinics with a deficiency of antithrombin, protein C or protein S, or with factor V Leiden were included when they had at least one relative with the same familial defect. Relatives with a prothrombotic defect were included regardless of their venous thrombosis disease status. As a representation of the normal population, partners or friends of the thrombophilic participants were included. For each participant, data were gathered at inclusion on previous venous events and thrombosis-related characteristics. During prospective follow-up, data were collected annually on the occurrence of risk situations and events (e.g., venous and arterial events, bleeding episodes and death). Inclusion was between March 1994 and September 1997, and prospective follow-up was until January 2001. The first chapter (Chapter I) includes the reports describing the results from the EPCOT study on the history of venous thrombosis before inclusion in the study in all participants (Chapter I.1), the risk of a first event during prospective follow-up in the relatives and control individuals with no history of venous thrombosis before study entry (Chapter I.2), and the risk of a second event during prospective follow-up in relatives with a first event before inclusion (Chapter I.3). In chapter I.4 we describe the results concerning a secondary aim: the risk of fetal loss during follow-up associated with inherited thrombophilia.

The international protein C investigation (IPCI)

Since 1985 a large family (n~800) with protein C deficiency has been studied which resides mainly in New England (USA) and Québec (Canada).[18] About 25% of the family members carry a C insertion in exon 6 of the protein C gene (3363C mutation), which is associated with protein C deficiency, and about 25% of the carriers have developed venous thrombotic disease.[18] A founder couple from France probably "imported" this 3363C mutation to

Québec around 1669.[22] In 1998, evidence was found for an unknown genetic defect influencing the risk of venous thrombosis in this family in interaction with protein C deficiency.[23] Several genes were tested as candidates for the interacting defect, including factor V Leiden and the prothrombin G20210A variant, but no evidence was found to support that any of these genes was the candidate gene.[24] For the prothrombin variant this was an unexpected finding as the carrier frequency in this family was high compared with the presence in the general Caucasian population (13% versus 1-4%).[25]
A subsequent investigation in which the probability of transmission of the prothrombin G20210A mutation was estimated among family members with or without the protein C mutation or a venous event even suggested a protective effect of the prothrombin mutation in the presence of the protein C mutation.[25] As the prothrombin G20210A mutation has been associated with increased prothrombin levels[14,26-28], we postulated a potential beneficial effect of increased thrombin-mediated activated protein C generation in carriers of the prothrombin G20210A mutation. By utilizing a new and highly sensitive assay for measuring the concentration of activated protein C in complex with protein C inhibitor as a measure of the level of activation of protein C, we tested this hypothesis (Chapter I.5).
To continue the search for unknown genes increasing the risk of venous thrombosis, we performed linkage analysis using DNA from plasma samples of the protein C deficient kindred.[29] Family members were genotyped for 375 autosomal markers, which were equally spaced across the genome with an average marker spacing of 9.4 cM (1 cM~1 million base pairs). Assuming that genes in close proximity to a marker will share the inheritance pattern of that particular marker, disease genes can be located by identifying those markers of which the inheritance pattern matches the inheritance pattern of the disease. Linkage analysis on 132 genotyped family members identified three potential candidate regions on chromosomes 10, 11 and 18 for a gene influencing the risk of venous thrombosis.[29]
A correct phenotype definition is crucial in performing linkage analysis. Therefore, we developed a questionnaire with detailed questions on venous thrombosis to create a more accurate phenotype definition. The questionnaire showed a high sensitivity for detecting a history of venous thrombosis in patients with venous thrombosis when comparing answers to the questionnaire with chart information.[30] This questionnaire was then distributed among more than 300 family members. However, as we earlier found less agreement between the self-reported history and the chart information regarding details on venous events in patients with an extensive history of venous thrombosis, chart review may still be needed in family members with multiple venous events for optimal ascertainment of a detailed thrombosis

history.[30] In addition, to extend the phenotype definition, ultrasound examinations were performed in family members to search for signs of venous thrombosis in the legs of those who were not known to have had venous thrombosis.[31]
Besides performing linkage analysis with venous disease as the outcome, which is either present or absent and usually infrequent, we extended the analysis to quantitative phenotypes related to hemostasis (e.g., factor VIII and prothrombin levels), which are measured on a continuous scale and probably closer connected to direct gene action. In Chapter II.1 the results are presented of an initial study, in which evidence was sought for a genetic basis for the variation in the plasma concentrations of several hemostatic measures. Chapter II.2 shows the results of a linkage analysis performed to identify loci influencing protein C activity as measured by concentrations of activated protein C in complex with two of its inhibitors α1-antitrypsin and protein C inhibitor, which showed high heritability in Chapter II.1.

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Chapter I

Inherited thrombophilia and the risk of venous thrombosis and fetal loss

Chapter I.1

Familial thrombophilia and lifetime risk of venous thrombosis

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Summary

We started a large multicenter prospective follow-up study to provide reliable risk estimates of venous thrombosis in families with various thrombophilic defects. This paper describes data collected at study entry on venous events experienced before study inclusion, i.e. the baseline data. All individuals (probands, relatives) registered in nine European thrombosis centers, with the factor V Leiden mutation, a deficiency of antithrombin, protein C or protein S, or a combination of these defects, were enrolled between March 1994 and September 1997. As control individuals, partners, friends or acquaintances of the thrombophilic participants were included. Incidence and relative risk of objectively confirmed venous thrombotic events (VTEs) prior to entry were calculated for the relatives with thrombophilia and the controls. Of the 846 relatives with thrombophilia (excluding probands), 139 (16%) had experienced a VTE with an incidence of 4.4 per 1000 person years. Of the controls, 15 of the 1212 (1%) controls had experienced a VTE with an incidence of 0.3 per 1000 person years. The risk of venous thrombosis associated with familial thrombophilia was 15.7 (95% CI 9.2-26.8) and remained similar after adjustment for regional and sex-effects (16.4; 95% CI 9.6-28.0). The highest incidence per 1000 person years was found in relatives with combined defects (8.4; 95% CI 5.6-12.2), and the lowest incidence was found in those with the factor V Leiden mutation (1.5; 95% CI 0.8-2.6). Considerable differences in the lifetime risk of VTE were observed among individuals with different thrombophilia defects.

Introduction

In developed countries, venous thrombosis occurs in 1 to 2 per 1000 individuals per year[1,2] and commonly manifests as deep vein thrombosis (DVT), with or without pulmonary embolism (PE).[3] Major complications in the clinical course of DVT are death from PE, development of a disabling post-thrombotic syndrome and recurrences.[2,4-6] Predisposing factors for venous thrombosis can be genetic or acquired, or both, and may lead to a life-long or temporary increase in the tendency to venous thrombosis (thrombophilia).[7] As the clinical expression varies between individuals who are heterozygous for the same genetic thrombotic defect, as shown within genotypically identical family members[8], venous thrombosis is believed to be a multicausal disease.[9,10] The hypothesis of multicausal pathogenesis is underlined by the finding that the risk of venous thrombosis is higher in families with inherited thrombophilia than in individuals with the same defect without a positive family history.[11,12] This difference probably is the result of interaction within these families of the inherited defect with other genetic or acquired risk factors.[11-13] Several

hereditary prothrombotic defects have been identified in the last four decades. The first was antithrombin deficiency, identified in 1965.[14] Since then, several other hereditary defects have been identified as risk factors for venous thrombosis, such as protein C deficiency, protein S deficiency, factor V Leiden and prothrombin G20210A.[15-18]

Rational guidelines for optimal treatment policies in families with inherited thrombophilia are lacking due to few available studies of sufficient size. Therefore, we started a large multicenter prospective follow-up study to determine the natural history of venous thrombosis in family members of symptomatic patients with at least one inherited prothrombotic defect.

With these risk estimates guidelines may be inferred for treatment and prevention of venous thrombosis in families with different kinds of inherited thrombophilia. Other endpoints such as arterial thrombotic disease (myocardial infarction, stroke), death from various causes, major hemorrhage and fetal loss were also studied. The European Prospective Cohort on Thrombophilia (EPCOT) study combines data on individuals with familial thrombophilia over a large geographical area of eight European nations and therefore is the largest cohort of individuals with inherited thrombophilia, thus providing reliable risk estimates.

This paper will describe the data collected at study entry on the occurrence of venous thrombosis in the EPCOT participants prior to entry, i.e. the baseline data on the history of venous thrombosis.

Study design

Participants and methods

A total of nine centers (Barcelona, Bologna, Frankfurt, Glasgow, Leiden, Malmö, Paris, Sheffield and Vienna) with long-standing interest in thrombophilia research from eight countries participated in the EPCOT study. We included patients referred to these specialist clinics to create a large cohort of individuals with deficiencies in natural anticoagulants, which are rare, and to address the risk of venous thrombosis found in thrombophilic families compared with patients with a similar thrombophilia defect without a family history. Each center enrolled all registered probands (first of a family in whom thrombophilia was detected) with a deficiency of antithrombin, protein C or protein S, or factor V Leiden, who had at least one relative with the same familial defect, and their registered relatives. Fewer individuals with factor V Leiden were included than would be expected based on the frequency of this mutation; however, the factor V Leiden mutation was identified more recently than the other defects, of which carriers were collected over the past 15 years. As controls, partners or, if there were none, friends or acquaintances of the thrombophilic participants were included. Controls were excluded if

they were known to have heritable thrombophilia or if they were related to a participant with an inherited thrombotic defect. There were no exclusion criteria for individuals with inherited thrombophilia: we included those who were symptomatic as well as those who were asymptomatic, and those who received anticoagulant treatment as well as those who did not. The study was approved by the Leiden University Hospital Medical Ethics Committee, and all participants in this study gave their informed consent.

Data collection

Inclusion took place between March 1994 and September 1997 with most of the participants (84% of controls and 88% of thrombophilic individuals) included in 1994 and 1995. Information was collected at inclusion, and annually during the prospective follow-up by standardized data collection forms. All data were collected by the responsible physician or another health professional at the participating centers by consulting another physician, by medical chart review or by telephone or mail contact with the patient. Completed forms were sent to the coordinating center with only the patient identifiers code to protect patient confidentiality. Data collected at study entry included information on general demographics, defect details (type, subtype, diagnostic methods, genetic confirmation), personal history with regard to thrombosis (dates, location, diagnostic test results), current medication (oral anticoagulants, oral contraceptives, hormone replacement therapy or other medication), other risk factors (e.g., obesity, varicose veins), obstetric history and family history of thrombosis. Data collection was identical for controls and thrombophilic individuals, except for items on the type and subtype of thrombophilia.

All centers performed the various assays according to their local protocol and participated in an external quality assessment scheme for thrombophilia testing. For the first two years this was the quality assurance scheme developed for the European Concerted Action on Thrombosis (ECAT/EQAS) (Leiden, The Netherlands) and for the subsequent years, the UK NEQAS quality assurance scheme (Sheffield, UK). Diagnostic criteria were based on those used in these centers, which for the deficiencies was based on repeated testing, and in some cases also on genotypic confirmation.

Recruitment was before identification of the G20210A mutation in the prothrombin gene. During prospective follow-up, we gathered information on the presence of this mutation as a second defect for 504 relatives and 424 probands. Thus we only have information on the prothrombin G20210A mutation as a second defect in 64% of the participants with inherited thrombophilia.

Analysis and statistics

For the analysis of the baseline data collected at study inclusion on the history of venous thrombotic events prior to study entry, we included only probands and relatives from families in which thrombophilia testing was done because of the occurrence of venous thrombosis in the proband or family, and not when this was done solely for research purposes or family planning. This restriction was to avoid selection bias and to stay as close as possible to the real-life situation of an individual from a symptomatic thrombophilia family asking a physician for advice.

We were interested in the number, type, age at onset, event-free survival, incidence and relative risk of venous thromboembolic events (VTEs) experienced before inclusion in the study. Only objectively confirmed events (by ultrasound, Duplex or venography for DVT, and by ventilation-perfusion scanning or angiography for PE) and confirmed events at other locations were counted as such; non-definite events, i.e. based on clinical or patient diagnosis were recorded but not considered in the analysis reported here, including superficial thrombophlebitis. Spontaneous venous thrombosis was defined as venous thrombosis without known precipitating risk factors (hospital admission, surgery, immobilization, plaster cast, uninterrupted travels over 8 hours, pregnancy, delivery). Thrombotic events in which the only risk factor was use of oral contraceptives were also labeled as spontaneous.

As probands were selected on having had venous thrombosis, we determined only the number and type of events, the age of onset and the age at which 50% of the probands had experienced venous thrombosis (median survival) prior to study entry. The probability of being free of events at any given age was analyzed by constructing Kaplan-Meier life tables. From these survival analyses we estimated the cumulative incidence of thrombosis with confidence intervals at age 30, 45 and 60.

The incidence of venous thrombotic events in relatives and controls was calculated by dividing the number of events by the total of observation years, i.e. the time between birth and the first event of interest, or until the end of study, i.e. the inclusion in the EPCOT study without a history of DVT, PE or other major event. The 95% confidence intervals (CIs) were calculated according to a Poisson distribution for the number of events.[19] Hazard ratios as an estimation of the relative risk were calculated by Cox-regression, with thrombosis as the dependent variable and presence of thrombophilia as an independent variable. Center (as stratum) and sex (as categorical variable) were entered in the Cox-regression model to adjust for regional and sex effects. Event-free survival, incidence and hazard ratios were calculated regardless of previous manifestations of superficial thrombophlebitis.

Results

A total of 2838 participants were enrolled in the cohort of whom 1626 had a thrombophilic defect (672 probands, 954 relatives of probands) and 1212 were controls (900 partners, 312 friends). In total, 600 probands and 846 relatives met the criteria that they were initially investigated because of thrombosis themselves or because of thrombosis in a family member.
The main characteristics at inclusion of probands, relatives and controls are depicted in Table 1. Among individuals with thrombophilia, men were slightly underrepresented (40% in probands and relatives). At study entry, 19 (10%) of the protein C deficient relatives, 43 (22%) of the protein S deficiency relatives, 32 (22%) of the antithrombin deficient relatives, 11 (5%) of the relatives with factor V Leiden and 22 (23%) of the relatives with combined defects received life-long anticoagulation.

Thrombotic history

Probands Of all included probands, 532 (89%) had experienced an objectively confirmed venous thrombosis prior to study entry (Table 2). The remaining 68 probands (11%) had been identified because of superficial thrombophlebitis (n=58), or were the first in the family in whom a defect was demonstrated with no personal but only a family history of venous thrombosis (n=10). Of all probands with a personal history of thrombosis, 288 (54%) had experienced one or more recurrencies before study entry. The mean age at the first venous thrombosis was 30 years (range 0-71) and ranged per type of defect from 26 years in patients with antithrombin deficiency to 33 years in patients with protein C deficiency or factor V Leiden (Table 2). Of all probands, 50% had experienced venous thrombosis before the age of 29 (Figure 1). Spontaneous venous thrombotic events, i.e. venous events without known precipitating risk factors or during use of oral contraceptives only, occurred in 220 of the 362 (61%) probands for whom this information was available.

Relatives and controls Of all included relatives, 139 (16%) had experienced an objectively confirmed venous thrombosis compared with 15 (1%) in the controls (Table 2) prior to study entry. The percentage of relatives with a venous event varied per type of defect from 6% in those with the factor V Leiden mutation to 29% in individuals with combined defects (Table 3). Recurrencies were present before baseline in 58 (42%) relatives with a history of venous thrombosis, and in only 2 (13%) of the controls with a history of venous thrombosis.
The mean age at the first venous thrombosis before study entry was 36 years (range 13-71) in the relatives and 41 years (range 24-68) in the

Table 1 *General characteristics of the probands, relatives and controls at baseline*

	Thrombophilic individuals		
	Probands	Relatives	Controls
All (n)	600	846	1212
Men (n)	237	339	627
Women (n)	363	507	585
PC deficiency (n)	126[a]	188	N/a
PS deficiency (n)	93	193	N/a
AT deficiency (n)*	102	145	N/a
FVL (n)	175[b]	225[c]	N/a
Combined defects (n)	104	95	N/a
PC deficiency-PS deficiency (n)	2	2	N/a
FVL-PC deficiency (n)	22[d]	22	N/a
FVL-PS deficiency (n)	23[d]	24	N/a
FVL-AT deficiency (n)	11	9	N/a
PT20210A-PC deficiency (n)	11	12	N/a
PT20210A-PS deficiency (n)	8	6[e]	N/a
PT20210A-AT deficiency (n)	5	6	N/a
PT20210A-FVL (n)	19[f]	13[g]	N/a
PT20210A-FVL-PC deficiency (n)	2	1	N/a
PT20210A-FVL-PS deficiency (n)	1	0	N/a
Age at inclusion (mean (range))	41 (2-78)	39 (0-91)	42 (3-87)
<18 yrs old (n)	5	73	36
18-45 yrs old (n)	355	470	695
>45 yrs old (n)	240	303	481

Abbrevations: PC=protein C, PS=protein S, AT=antithrombin, FVL=factor V Leiden, PT20210A= prothrombin G20210A, N/A=not applicable. [a]2 were homozygous [b]31 were homozygous [c]13 were homozygous [d]1 was homozygous for FVL [e]1 was homozygous for PT20210A [f]4 were homozygous for FVL [g]1 was homozygous for FVL and 1 was homozygous for PT20210A *Six probands and 14 relatives showed only low antithrombin activity, 2 relatives were identified by DNA-testing only, and 5 probands and 7 relatives had only activity levels measured.

controls (Table 2). Per type of defect, the age of onset ranged from 28 years in relatives with combined defects to 42 years in relatives with protein C deficiency (Table 2).

Spontaneous venous events occurred before study entry in 54 of the 90 (60%) relatives and in 7 of the 14 (50%) controls for whom this information was available, with thrombotic events occurring during use of oral contraceptives considered as spontaneous events.

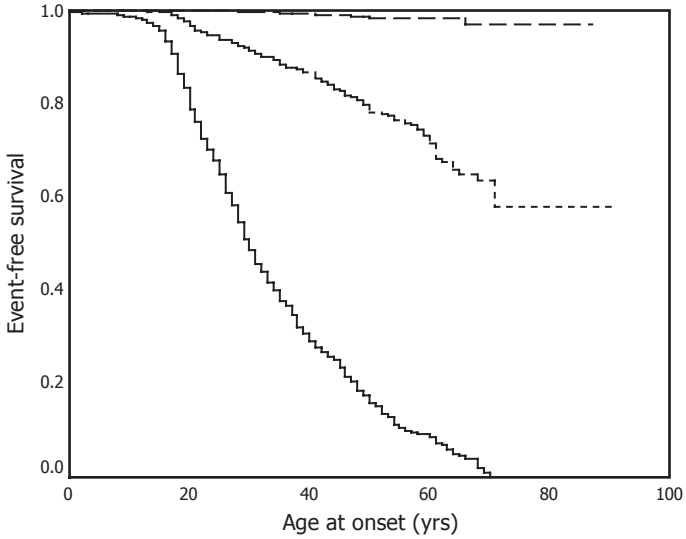
The incidence of venous events experienced before study entry in relatives

Table 2 *Description of objectively confirmed venous events before study entry*

	Thrombophilic individuals		
	Probands (n=600)	Relatives (n=846)	Controls (n=1212)
Individuals with venous events before baseline (n)	532	139	15
DVT (n)	359	89	9
PE (n)	154	45	3
Other VT (n)	19	5	3
PC deficiency (n)	110	22	N/a
PS deficiency (n)	84	50	N/a
AT deficiency (n)	90	25	N/a
FVL (n)	152[a]	14[b]	N/a
Combined defects (n)	96[c]	28	N/a
Age at onset all major events (mean (range))	30 (0-71)	36 (13-71)	41 (24-68)
PC deficiency	32 (11-71)	42 (23-71)	N/a
PS deficiency	29 (2-68)	37 (13-71)	N/a
AT deficiency	26 (0-53)	34 (17-64)	N/a
FVL	33 (14-68)	38 (17-61)	N/a
Combined defects	28 (3-69)	28 (13-59)	N/a

Abbreviations: DVT= deep venous thrombosis, PE= pulmonary embolism with or without DVT, VT= venous thrombosis, PC=protein C, PS=protein S, AT=antithrombin, FVL=factor V Leiden, N/a=not applicable. [a]30 were homozygous [b]2 were homozygous [c]6 were homozygous for FVL

Figure 1 *Venous event-free survival of the probands (solid line), relatives (short dashed line) and controls (long dashed line) until study entry*



was 4.4 (95% CI 3.7-5.2) per 1000 person years and in controls 0.3 (95% CI 0.2-0.5) per 1000 person years (Table 3). Per type of thrombophilia, the incidence was lowest in the relatives with the factor V Leiden mutation (1.5 per 1000 person years) and highest for those with combined defects (8.4 per 1000 person years) (Table 3). The incidence regarding factor V Leiden did not change after exclusion of homozygous individuals. The incidence was higher in men in the relatives (6.2 per 1000 person years for men and 3.2 per 1000 person years for women), but similar for both men and women in the controls (0.3 per 1000 person years for men and 0.2 per 1000 person years for women) (Table 3).
The probability of being free of venous events before study entry in the thrombophilic individuals (probands excluded) was 91% (95% CI 89-93%) at age 30, 82% (95% CI 79-86%) at age 45 and 70% (95% CI 65-76%) at age 60 (Figure 1). Per type of defect, the probability of being free of venous events at age 45 was 87% with protein C deficiency, 74% with protein S deficiency, 80% with antithrombin deficiency, 94% with factor V Leiden and

Table 3 *Incidence per 1000 person years of venous events before study entry in the controls and the relatives*

	All (n)	Events (n (%))	Person years (yrs)	Incidence (per 1000 yrs (95% CI))
Controls	1212	15 (1)	51079	0.3 (0.2-0.5)
Men	627	9 (1)	26746	0.3 (0.2-0.6)
Women	585	6 (1)	24333	0.2 (0.1-0.5)
Age at inclusion >18 years*	1171	15 (1)	29504	0.5 (0.3-0.8)
Age at inclusion >45 years**	477	6 (1)	5712	1.1 (0.4-2.3)
Relatives	846	139 (16)	31660	4.4 (3.7-5.2)
PC deficiency	188	22 (12)	7059	3.1 (2.0-4.7)
PS deficiency	193	50 (26)	7059	7.1 (5.3-9.3)
AT deficiency	145	25 (17)	5034	5.0 (3.2-7.3)
FVL	225	14 (6)	9186	1.5 (0.8-2.6)
Combined defects	95	28 (29)	3322	8.4 (5.6-12.2)
Men	339	78 (23)	12634	6.2 (4.9-7.7)
Women	507	61 (12)	19026	3.2 (2.5-4.1)
Age at inclusion >18 years*	743	126 (17)	16893	7.5 (6.2-8.9)
Age at inclusion >45 years**	260	39 (15)	3593	10.9 (7.7-14.8)

Abbreviations: PC=protein C, PS=protein S, AT=antithrombin, FVL=Factor V Leiden, CI=confidence interval. *Only person years above 18 years were counted. Individuals older than 18 years at baseline who had events before age 18 were excluded. **Only person years above 45 years were counted. Individuals older than 45 years at baseline who had events before age 45 were excluded.

67% with combined defects. In the controls, the probability of being free of venous thrombosis at age 30, 45 and 60 was, respectively, 100%, 99% (95% CI 98-100%) and 98% (95% CI 97-99%) (Figure 1).
The risk of venous thrombosis derived from the incidences of venous events experienced before study entry was 16 times higher in the relatives with thrombophilia compared with the controls (relative risk of 15.7; 95% CI 9.2-26.8) and remained similar after adjustment for center and sex (relative risk of 16.4; 95% CI 9.6-28.0) (Table 4). Per type of thrombophilia the relative risk differed greatly: the highest risk was found in the relatives with combined defects. For the relatives with single defects, the risk was highest in relatives with protein S deficiency (32.4; 95% CI 16.7-62.9) and lowest in the relatives with the factor V Leiden mutation (4.3; 95% CI 1.9-9.7) (Table 4). Exclusion of homozygous individuals with the factor V Leiden mutation did not affect these estimates. The relative risk was higher in men than in women: 18.1 (95% CI 9.0-36.3) and 13.9 (95% CI 6.0-32.4), respectively (Table 4).

Discussion

To obtain reliable estimates of the risk of venous thrombosis associated with familial thrombophilia caused by various defects, we started a prospective collaborative multinational study, including 1626 individuals with inherited thrombophilia and 1212 controls from eight European countries.

Table 4 *Relative risk of venous events before study entry in the relatives*

	Crude relative risk*	Adjusted** relative risk
All relatives versus controls	15.7 (9.2-26.8)	16.4 (9.6-28.0)
PC deficiency	11.1 (5.7-21.4)	11.3 (5.7-22.3)
PS deficiency	26.1 (14.7-46.5)	32.4 (16.7-62.9)
AT deficiency	19.0 (10.0-36.1)	17.5 (9.1-33.8)
FVL	5.2 (2.5-10.8)	4.3 (1.9-9.7)
Combined defects	32.0 (17.1-60.0)	46.7 (22.5-97.1)
Men	19.2 (9.6-38.4)	18.1 (9.0-36.3)
Women	13.8 (5.9-31.8)	13.9 (6.0-32.4)
Age at inclusion >18 years#	14.4 (8.4-24.6)	14.4 (8.4-24.6)
Age at inclusion >45 years##	10.2 (4.3-24.0)	10.3 (4.3-24.4)

Abbreviations: PC=protein C, PS=protein S, AT=antithrombin, FVL=Factor V Leiden. *For every defect, we compared relatives with the defect with all controls. **Adjusted for regional and sex-effects. For the relative risk per sex, the relative risk was only adjusted for regional effects. #Only person years above 18 years were counted. Individuals older than 18 years at baseline who had events before age 18 were excluded. ##Only person years above 45 years were counted. Individuals older than 45 years at baseline who had events before age 45 were excluded.

Data collected at study entry on the history of venous thrombosis prior to study inclusion showed a 16 times increased risk of venous thromboembolic events for the individuals with inherited thrombophilia (only relatives of probands included) compared with the normal population (crude relative risk of 15.7 (95% CI 9.2-26.0), adjusted relative risk of 16.4 (95% CI 9.6-28.0) adjusted for sex and regional effects). The incidence of venous events before study entry was 4.4 per 1000 person years in the relatives, compared with 0.3 per 1000 person years in the controls.

The highest incidence of events and the lowest age at onset before study entry were found in the relatives with combined defects (8.4 per 1000 person years; mean age at onset 28 years), as has been described by other authors.[11,20-25] For single defects we found the highest risk with protein S deficiency (7.1 per 1000 person years), and the lowest for factor V Leiden (1.5 per 1000 person years). Although there is one report that protein S deficiency confers the highest risk of venous thromboembolism[26], several others have shown that the greatest venous thromboembolic risk is associated with antithrombin deficiency.[23,24,27,28] Whether there are real differences in the venous thrombotic risk in respect of antithrombin and protein S deficiency remains unresolved. The absence of a difference in our study might reflect differences in the distribution of, yet unknown, interacting second defects or the presence of the prothrombin G20210A mutation for which 36% of the participants could not be tested. It is, however, noteworthy, that a population study, in which selection or referral bias was excluded, also did not find a higher risk for antithrombin deficiency.[29] Another possible explanation for these conflicting results is that since antithrombin deficiency was the first thrombophilia to be discovered, affected individuals could have received prophylaxis more frequently than individuals with other types of thrombophilia. However, when the year of diagnosis of thrombophilia was taken as follow-up endpoint instead of the age at which individuals were without venous events at study entry, the incidence was only slightly higher for individuals with antithrombin deficiency: 5.9 per 1000 person years (95% CI 3.8-8.7).

The annual risk of thrombophilia-associated venous thrombosis before study entry was sex-dependent: the incidence was higher in male relatives, but similar for both male and female controls. As many venous thrombotic events in young women can be attributed to oral contraception, the low percentage of asymptomatic thrombophilic women using oral contraceptives (16%; age 15-35) compared with asymptomatic female controls (37%; age 15-35) offers a likely explanation for a lower risk in women with thrombophilia compared with control women. Another explanation is that female relatives were referred to a thrombosis clinic for investigation before hormone prescription or pregnancy, whereas men were only referred when they were symptomatic. Labelling events as provoked or unprovoked is difficult and mostly dependent

on the researcher's definition of a provoked event, e.g., we labeled events occuring during oral contraceptive use as unprovoked, as oral contraceptive use is a generally weak and very common risk factor during which anticoagulation treatment is mostly not considered. Studies including detailed, reliable information on risk factors could give insight in the risk of venous thrombosis associated with these risk factors.

We have included over 1500 individuals with familial thrombophilia from eight European countries, so our study yields reliable and generalizable results. It should be noted, however, that in this study follow-up was counted only for those entered in the cohort, i.e., individuals with any of these defects who died before the start of the study were not included. This implies that we may have underestimated the risk of thrombosis. However, in previous studies we have shown that the mortality of antithrombin deficiency[30], protein C deficiency[31] and factor V leiden[32] does not exceed the population risk. We also only counted objectively confirmed manifestations of venous thrombosis to avoid selection bias, which means that we may have excluded inadequately diagnosed or missed events, and thus could have underestimated the risk. However, only a fraction of all reported events were not objectively confirmed. We also did not have full details on thromboprophylaxis prior to recruitment. The incidence of venous thromboembolic events may thus have been reduced in the relatives if short-term thromboprophylaxis was used to cover surgery, trauma or pregnancy. After diagnosis of a hereditary thrombotic defect, thromboprophylaxis may be more likely to be offered even to asymptomatic relatives and oral contraception with estrogen-containing preparations is discouraged at least in some countries. However, incidences per defect were only slightly higher or remained similar to the incidences shown in Table 3 when the year of diagnosis of thrombophilia was taken as the study end-point instead of the age at which individuals were without venous events at study entry. The risk of venous thrombosis might have been overestimated when mostly symptomatic relatives were referred to a thrombosis clinic for investigation. In addition, the risk for individuals with single defects might have been overestimated when they were carriers of the prothrombin G20210A mutation but could not be tested for this particular mutation in our study.

It is important to note that our results concern individuals from thrombophilic families registered at specialized clinics, and hence the results may be generalized to such individuals, but not to unselected individuals with the same defect, as we have previously shown that these individuals have a lower risk of thrombosis.[11,12]

While more detailed data will result from the prospective follow-up, this study showed that individuals with familial thrombophilia have an increased risk of venous thrombosis with a high risk of spontaneous thrombosis and

recurrencies. Since 70% of the individuals with thrombophilic defects were free of thrombosis at age 60, it is unlikely that long-term anticoagulation started at a young age would have benefits outweighing the risks of this treatment especially in those with a single genetic prothrombotic defect.

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Chapter I.2

Risk of a first venous thrombotic event in carriers of a familial thrombophilic defect

The European Prospective Cohort on Thrombophilia (EPCOT)

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Summary

Reliable risk estimates for venous thrombosis in families with inherited thrombophilia are scarce but necessary for determining optimal screening and treatment policies. In the present analysis, we determined the risk of a first venous thrombotic event in carriers of a thrombophilic defect (i.e. antithrombin-, protein C- or protein S deficiency, or factor V Leiden). The asymptomatic carriers had been tested prior to this study in 9 European thrombosis centers because of a symptomatic carrier in the family, and were followed prospectively for 5.7 years on average between March 1994 and January 2001. Annually, data were recorded on the occurrence of risk situations for venous thrombosis and events (e.g., venous thrombosis, death). Twenty-six of the 575 asymptomatic carriers (4.5%) and 7 of the 1118 controls (0.6%) experienced a first deep venous thrombosis or pulmonary embolism during follow-up. Of these events, 58% occurred spontaneously in the carriers compared with 43% in the controls. The incidence of first events was 0.8% per year (95% CI 0.5-1.2) in the carriers compared with 0.1% per year (95% CI 0.0-0.2) in the controls. The highest incidence was associated with antithrombin deficiency or combined defects, and the lowest incidence with factor V Leiden. The incidence of venous events in asymptomatic individuals from thrombophilic families does not exceed the risk of bleeding associated with long-term anticoagulant treatment in the literature (1-3%).

Introduction

Venous thrombosis has an overall incidence of about 1-2 per 1000 individuals per year and is a serious disorder with potential major complications such as death from pulmonary embolism, recurrences and the development of a disabling post-thrombotic syndrome[1-3]. It has been postulated that not a single risk factor, but interaction between multiple genetic and environmental risk factors is a prerequisite for venous thrombosis to develop[4-6]. Currently, several genetic risk factors are known to increase the risk of venous thrombosis: deficiencies in the anticoagulation factors protein C, protein S and antithrombin, and the factor V Leiden and prothrombin G20210A mutations [7-11]. In accordance with the multicausal nature of thrombosis, it was found that the presence of a family history of venous thrombosis increases the thrombotic risk in individuals with protein C deficiency or factor V Leiden, most likely due to the concomitant presence of other genetic or environmental risk factors within the family[6,12,13].

A prerequisite to determining the optimal clinical approach, e.g., with regard to screening or (long-term) prophylactic anticoagulant treatment in families with inherited thrombophilia is to define the absolute risk of venous

thrombosis, and to weigh this risk against the risk of bleeding complications associated with prophylactic treatment. To obtain valid risk estimates of the absolute risk of venous thrombosis in families with inherited thrombophilia, we started the European Prospective Cohort on Thrombophilia (EPCOT) study. The EPCOT study, by combining data from nine centers, is the largest prospective cohort of individuals with deficiencies of protein C, protein S and antithrombin, and factor V Leiden. In this paper, we present data on the risk of a first venous thromboembolic event associated with inherited thrombophilia in relatives of probands from thrombophilic families who were asymptomatic at the time of inclusion in the study.

Methods

Participants

The design of the study has been described in detail previously[14]. In short, inclusion of the participants took place between March 1994 and September 1997 with prospective follow-up until January 2001. Nine centers from eight countries (Austria, France, Germany, Italy, Spain, Sweden, The Netherlands and the United Kingdom) participated. Each center enrolled all registered probands (first of a family in whom thrombophilia was detected) with a deficiency of protein C, protein S or antithrombin, or factor V Leiden, and their registered relatives with one of these defects. Healthy partners, or, if there were none, friends or acquaintances of participating individuals with inherited thrombophilia were included as controls. Controls were excluded if they were known to have heritable thrombophilia, or if they were related to a participant with an inherited thrombotic defect. Controls were, however, not tested for any of the defects under study after study entry. All participants gave informed consent. Data were collected at baseline, and annually during follow-up. At study entry data were collected on subjects' general demographics, hereditary defect (type, subtype (if available), levels (if available), information on DNA testing), current medication and current risk factors for thrombosis, history of thrombosis, obstetric history and family history of thrombosis. The data recorded at follow-up included the occurrence of risk situations (surgery, hospital admission, plaster casts, prolonged bed rest (>2 weeks), traveling (>8 hours), details on pregnancies, medication) and on outcome events (e.g., venous thrombosis, hemorrhage, death). Completed forms were sent to the coordinating center with only the patient identifiers code to protect patient confidentiality.

From a total of 1626 individuals with thrombophilic defects and 1212 control subjects, this paper includes only relatives of probands who had not previously experienced any venous thrombotic event (deep venous thrombosis (DVT), pulmonary embolism (PE), or superficial thrombophlebitis (STP)) before

inclusion in the study, and who did not receive long-term prophylactic oral anticoagulant treatment during prospective follow-up (defined as treatment for at least 1 year without interruptions). Twenty thrombophilic individuals received long-term prophylactic oral anticoagulant treatment: 10 for prevention of venous thrombosis and 10 for a personal history of arterial disease. Eight controls received long-term anticoagulation treatment for an arterial indication. In addition, we only included relatives of probands tested because of venous thrombosis or a positive family history. Families in which thrombophilia was detected because of screening before hormone prescription or research purposes were not included, in an effort to stay as close as possible to the real-life situation of an individual from a symptomatic thrombophilia family asking a physician for advice.
Recruitment was before description of the G20210A mutation in the prothrombin gene, but during prospective follow-up we gathered information on the presence of this mutation as an additional defect.

Quality assessment thrombophilia testing and verification of events

The participating centers performed the various assays according to their local protocol and participated in an external quality assessment scheme for thrombophilia testing. For the first two years this was the quality assurance scheme developed for the European Concerted Action on Thrombosis (ECAT/EQAS) (Leiden) and for the subsequent years the UK National External Quality Assessment Service (NEQAS) (Sheffield, UK).
Per center, a local investigator verified all events in prospective follow-up. In case of death, the autopsy data were collected, if available. An adjudication committee scored all reported events independently after receiving all the relevant medical information from each center, but in case of doubt or when no consensus was reached at first, further information was asked for (e.g., actual test results). Committee members were not blinded for whether a person was a thrombophilia carrier or control, but were unaware of the type of defect of the carriers. Venous thromboses were classified as definite venous events when they were objectively confirmed, i.e. for DVT by ultrasound (duplex, Doppler), impedance plethysmography, venography or leg symptoms plus definite PE, and for PE by angiography, high probability ventilation-perfusion-scan, spiral computerized tomography or autopsy. STPs were considered definite events when diagnosed by a physician. For the analysis reported here, we included only definite venous events. Consensus on the classification of the venous events, i.e. that a majority of the steering committee members agreed, was reached in 67 of the 72 (93%) reported events during prospective follow-up (47 DVTs or PEs, 21 STPs, 4 major hemorrhages). Of the remaining 5 events, which were classified as definite

DVT or PE (n=4) or no PE (n=1), consensus was reached after additional information was provided (n=4) or after reconsideration (n=1). For controls, immediate consensus was reached in 88% of the reported events (15 out of 17) and in thrombophilic subjects in 95% of the reported events (52 out of 55).

Analysis and statistics

We calculated the annual absolute risk (incidence) and relative risk of a first DVT or PE for various groups in the cohort. The incidence of venous thrombotic events was calculated by dividing the number of events by the total of observation-years (follow-up time). Follow-up time was the time between inclusion and the event of interest, death or the last date of follow-up (the end of the study or last date before loss-to-follow-up), whichever occurred first. A DVT or PE was considered first event only when not preceded by a STP. If a PE followed a DVT within 3 months, these events were considered a single event. The 95% confidence intervals (95% CIs) were calculated according to a Poisson distribution for the number of events [15]. Hazard ratios as estimation of the relative risk of venous thrombosis were calculated by Cox-regression with venous thrombosis as the dependent variable and presence or absence of thrombophilia as independent variable. Center, age (as stratum: age <45 or ≥45), and sex were entered in the Cox-regression model to adjust for center, age and sex effects.

Results

We collected prospective data on 575 relatives with a thrombophilic defect and 1118 controls (825 partners, 293 friends), all without a thrombotic event prior to inclusion and not on long-term oral anticoagulant treatment. The total follow-up time was 3283 years in the individuals with thrombophilia (mean 5.7; range 0.2-7.3) and 6289 years in the controls (mean 5.6; range 0.7-7.2). During follow-up, 20 thrombophilic subjects and 64 controls were lost to follow-up (complete follow-up in 95%), and 8 thrombophilic individuals and 12 controls died. The causes of death in the carriers were heart disease (n=3), suspected pulmonary embolism (n=1), and other causes (n=4). Causes of death in controls were not related to venous thrombosis (cancer (n=4), myocardial infarction (n=4), accidents in traffic or at work (n=3), or surgery (n=1)). The main characteristics at inclusion are shown in Table 1. A preponderance of the thrombophilic individuals was female (62%), whereas sex was distributed more equally distributed among the controls.

Table 1 *General characteristics at inclusion*

	Thrombophilic individuals	Controls
All (n)	575	1118
Men (n)	214	588
Women (n)	361	530
OCC use, age 10-50 (n/total n)*	63/265	153/360
HRT use, age ≥50 (n/total n)	17/77	47/159
PC deficiency (n)	143	N/a
PS deficiency (n)	107	N/a
AT deficiency (n)	96	N/a
FVL (n)	173[a]	N/a
Combined defects (n)	56	N/a
PC-PS (n)	1	N/a
FVL-PC (n)	11	N/a
FVL-PS (n)	15	N/a
FVL-AT (n)	6	N/a
FVL-PT20210A (n)	8[b]	N/a
PT20210A-PC (n)	9	N/a
PT20210A-PS (n)	4[c]	N/a
PT20210A-AT (n)	2	N/a
Mean age at inclusion (years (range))	35 (0-91)	41(3-87)
Mean BMI (kg/m^2 (range))**	23 (13-42)	24 (13-39)
Cancer ever (n (%))	8 (1)	12 (1)

*Abbreviations: OCC=oral contraceptives, HRT=hormone replacement therapy, PC=protein C, PS=protein S, AT=antithrombin, FVL= factor V Leiden, PT20210A=prothrombin G20210A, N/a=not applicable, BMI= body mass index. [a]10 were homozygous [b]1 was homozygous for factor V Leiden [c]1 was homozygous for prothrombin G20210A *The oral contraceptives contained estrogen in 34/265 (13%) thrombophilic women, and 139/360 (39%) of the control women. **Information on BMI was available for 574 thrombophilic individuals and 1117 controls.*

Risk of a first event

Of the 575 thrombophilic individuals, 26 thrombophilic subjects experienced a first DVT or PE during follow-up (4.5%) (1 relative had a mesenteric vein thrombosis) compared with 7 of the 1118 controls (0.6%) (Table 2). The events occurred spontaneously, i.e. did not occur after exposure to a known acquired risk factor, in 15 thrombophilic individuals (58%) and 3 controls (43%). The risk factors present at the time of the event in the remaining 11 thrombophilic individuals were hospitalization (n=3), hormone replacement therapy (n=2), infection (n=2), pregnancy (n=2), cancer (n=1) and oral contraceptives (n=1), and in the 4 remaining controls surgery (n=2), hospitalization (n=1) and cancer (n=1). The incidence of a first DVT or PE was higher in the thrombophilic individuals than in the controls, respectively, 0.8% per year (95% CI 0.5-1.2) compared with 0.1% per year (95% CI 0.0-0.2)

(Table 2) with a relative risk of 9.0 (95% CI 3.8-21.1), adjusted for sex, age at entry and center effects (crude relative risk: 7.3; 95% CI 3.2-16.8). The incidence of a STP as first event was 0.5% per year (95% CI 0.3-0.8) in the thrombophilic subjects, and 0.1% per year (95% CI 0.0-0.2) in the controls (Table 2) with a relative risk of 5.2 (95% CI 2.0-13.7), adjusted for sex, age at entry and center effects (crude relative risk: 5.3; 95% CI 2.1-13.5).
The annual incidence of a first DVT or PE was highest for thrombophilic individuals with antithrombin deficiency (1.7%/year; 95% CI 0.8-3.3) or combined defects (1.6%/year; 95% CI 0.5-3.7) and lowest in thrombophilic individuals with the factor V Leiden mutation (0.1%/year; 95% CI 0.0-0.6) (Table 2). Unfortunately, the numbers per type of defect were too small to estimate the risks by age and sex. Our database contained subtype information (or sufficient level information to determine the subtype) of 120 protein C deficient individuals, 97 individuals with protein S deficiency and 76 individuals with antithrombin deficiency. When we included in the analysis only individuals known to have subtype I (103 with protein C deficiency, 68 with protein S deficiency, and 59 with antithrombin deficiency), the risk of a major event was similar to the risk in Table 2. The risk of thrombosis in those with a type II defect or type III protein S defect was not lower than in those with type I abnormalities, although the number of individuals (n=63) with type II or III defects was low (results not shown).
In men with thrombophilia, the annual incidence of a first DVT or PE was higher (1.4%/year; 95% CI 0.8-2.2) compared with thrombophilic women (0.5%/year; 95% CI 0.2-0.9) (Table 2). In controls, the incidences of a first DVT or PE did not differ between the sexes (Table 2). The percentage of women above the age of 50 using hormone replacement therapy was slightly lower in thrombophilic women (22%) compared with controls (30%), whereas a much lower percentage of women with inherited thrombophilia used estrogen-containing oral contraceptives (13%; age 10-50 years) compared with the control women (39%; age 10-50 years). The incidence of a first DVT or PE in thrombophilic women who did not use oral contraceptives (age 10-50 years) was 0.4%/year (95% CI 0.1-0.9) compared with 0.5%/year (95% CI 0.0-2.9) in women using estrogen-containing oral contraceptives.
The first DVT or PE occurred about 20 years earlier in the thrombophilic individuals than in the controls (Table 2). The mean age at onset was ~40 years for individuals with protein C-, protein S- or antithrombin deficiency or combined defects and 63 years for those with factor V Leiden in comparison to 63 years in the controls (Table 2).
The risk of a first DVT or PE was 1.0% per year (95% CI 0.6-1.6) among the 285 thrombophilic individuals who encountered acquired risk factors during prospective follow-up and who did not receive short-term anticoagulation during these risk situations (i.e. surgery, hospitalization, bed rest for more

Table 2 *Incidence (%/year) and age at onset of a first venous thrombosis*

	Total subjects	DVT/PE			
	n	n	Age onset, years (range)	Person years, n	Incidence, %/year (95% CI)
Thrombophilic individuals	575	26	40 (20-65)	3194.4	0.8 (0.5-1.2)
Protein C deficiency	143	6	41(22-65)	833.2	0.7 (0.3-1.6)
Protein S deficiency	107	5	38 (28-63)	619.1	0.8 (0.3-1.9)
Antithrombin deficiency	96	9	39 (21-58)	520.6	1.7 (0.8-3.3)
Factor V Leiden	173	1*	63	902.1	0.1 (0.0-0.6)
Multiple defects	56	5*	36 (20-62)	319.4	1.6 (0.5-3.7)
Males	214	16	37 (20-65)	1162.0	1.4 (0.8-2.2)
Females	361	10	44 (21-65)	2032.4	0.5 (0.2-0.9)
Age at inclusion 18-45 yrs	339	18	32 (21-47)	1880.1	1.0 (0.6-1.5)
Age at inclusion above 45 yrs	146	7	61 (51-65)	793.2	0.9 (0.4-1.8)
Controls	1118	7	63 (38-84)	6269.6	0.1 (0.0-0.2)
Males	588	2	58 (57-59)	3303.0	0.1 (0.0-0.2)
Females	530	5	64 (38-84)	2966.6	0.2 (0.1-0.4)
Age at inclusion 18-45 yrs	652	1	38	3601.8	0.0 (0.0-0.2)
Age at inclusion above 45 yrs	426	6	67 (57-84)	2445.7	0.2 (0.1-0.5)

Abbreviations: DVT=deep venous thrombosis, PE=pulmonary embolism, CI=confidence interval
*All were heterozygous.

than 13 days, plaster cast, cancer, pregnancy or traveling for more than 8 hours). Table 3 shows the frequency of venous events associated with the presence of acquired risk factors during prospective follow-up for which no short-term prophylactic anticoagulant treatment was provided. Cancer, although the number of individuals was small, and pregnancy appear to be the risk factors during which most secondary venous events occurred in the thrombophilic individuals. The incidence of a first DVT or PE in the 156 thrombophilic individuals who did not encounter acquired risk factors for which they could have received short-term anticoagulation during prospective follow-up was, however, similar: 0.8% per year (95% CI 0.3-1.7).
A total of 134 thrombophilic individuals (23%) received short-term prophylactic anticoagulation during one or more risk situations: surgery (n=95), pregnancy (n=43; 13 during puerperium only; range start treatment during pregnancy: week 5-week 36), plaster cast (n=13), traveling (n=9) and after twisting a knee or an ankle (n=2). No venous events occurred during any

of these risk situations under short-term prophylactic anticoagulant treatment. Of the controls, 115 (10%) also received short-term anticoagulant treatment during risk situations: during surgery (n=107), pregnancy (n=4; 3 during puerperium only), plaster cast (n=12), traveling (n=1) and after twisting a knee (n=1) or during chronic enteritis (n=1).

Table 3 *Number of first venous events per risk situation for which subjects did not receive short-term prophylactic anticoagulation*

	Thrombophilic individuals			Controls		
	n	Situations, n	DVT/PE, n (%)	n	Situations, n	DVT/PE, n (%)
Travel (>8 hours)	260	504*	0 (0%)	567	1244*	0 (0%)
Surgery/immobilization**	143	176	3 (2%)	290	407	2 (0%)
Plaster cast	27	33	0 (0%)	59	71	0 (0%)
Cancer#	10	10	1 (10%)	17	17	1 (6%)
Pregnancies	24	28	2 (7%)	61	75	0 (0%)

Abbreviations: DVT=deep venous thrombosis, PE=pulmonary embolism. *The number of follow-up years in which individuals reported to have traveled at least once for more than 8 hours. **Immobilization is defined as a hospital stay, or bed rest for at least 14 days at home. #Defined as malignancy still present or developed during prospective follow-up. Nine (4 thrombophilic individuals, 5 controls) received anticoagulation during and after surgery for cancer.

Discussion

We conducted a large prospective follow-up study in nine centers in eight countries on 1626 probands and relatives, and 1212 controls to obtain valid estimates of the risk of venous thrombosis in families with inherited thrombophilia. In the present report, we describe the risk of a first venous thromboembolic event in 575 thrombophilic individuals and 1118 control subjects who were followed up to 7 years (mean follow-up 5.6 years) and who were asymptomatic and not on long-term anticoagulant treatment at study inclusion.

Among the thrombophilic individuals, the annual risk of a first DVT or PE was 0.8% per year. The incidence was lowest for thrombophilic individuals with factor V Leiden (0.1%/year) and highest for thrombophilic subjects with antithrombin deficiency (1.7%/year), individuals with combined defects (1.6%/year) and men (1.4%/year). DVTs and PEs occurred spontaneously in 58% of the thrombophilic individuals and 43% of the controls. The annual risk was not increased in thrombophilic individuals who encountered additional acquired risk situations (e.g., surgery, immobilization, pregnancy, plaster cast) during which no short-term anticoagulation treatment was prescribed. As

134 thrombophilic individuals did receive short-term anticoagulation during risk situations, mainly during pregnancy and surgery, this finding could be the result of the intention of doctors to treat those with a high risk of venous thrombosis only in definite high-risk situations.

The strongest point of this study is its size: deficiencies of natural anticoagulants are rare, and large prospective studies are therefore scarce. We excluded events that were not objectively confirmed; as these were few and equally distributed over thrombophilic individuals and controls (of the DVTs and PEs 12% were non-definite in the carriers compared with 14% in the controls) this could not have affected our results. The results of this study should not be generalized to all individuals with thrombophilia, as we focused on asymptomatic subjects with familial thrombophilia, to which patient group these results apply. We have shown previously that the risk of thrombosis is higher in selected individuals from families with a clear familial thrombophilia, than in unselected individuals without a strong family history, regardless of the defect[12,13]. Other studies on the risk of a first venous thrombotic event in carriers of a hereditary defect[16-22] reported annual incidences of venous thrombosis ranging from 0.4% to 2.5% for protein C deficiency, 0.1% to 3.2% for protein S deficiency, and 0.9% to 2.9% for antithrombin deficiency. For factor V Leiden, the reported incidences ranged from 0.1% to 0.7% per year [17,19,23-25]. The risks found in the present study could have been underestimated due to preventative measures taken by the treating physicians, who were aware of the presence of a thrombophilic defect, to reduce the risk of venous thrombosis. However, the risk for individuals with single defects might have been overestimated when they carried the prothrombin G20210A mutation but were not tested for this particular mutation in our study. We were able to gather information on the presence of this mutation as an additional defect for 360 of the 575 thrombophilic individuals (63%).

Although the risk of a first DVT or PE was increased in thrombophilic individuals, the risk was still quite low and did not outweigh the reported risk of 1-3% of hemorrhagic complications in patients with prolonged anticoagulant therapy with target INRs with a lower limit of 2.0[26,27]. Therefore, long-term prophylactic anticoagulant treatment does not seem beneficial in asymptomatic relatives of symptomatic probands with inherited thrombophilia, although future prophylactic treatment options with a lower risk of bleeding might change this. However, there might be a need for a more stringent thromboprophylactic treatment policy during temporary periods of increased thrombotic risk as no DVT or PE occurred in thrombophilic individuals during risk situations in which prophylactic anticoagulation was used. In the thrombophilic individuals, 42% of all DVTs or PEs occurred in the presence of a known risk situation and could therefore have been prevented.

Whether screening or short-term prophylaxis would be beneficial is difficult to

conclude from our results, as the low risk of thrombosis might be the result of screening and a policy aimed at preventing thrombosis, as can be deduced from the low number of women with estrogen-containing oral contraceptives and the high number of women with prophylactic anticoagulant therapy during pregnancy or puerperium. Further studies should elucidate whether a more stringent policy of thromboprophylaxis in risk situations could further reduce the risk of thrombosis, which should in particular focus on individuals with antithrombin deficiency and combined defects, who had the highest risk of thrombosis.

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Chapter I.3

Recurrence rate after a first venous thrombosis in patients with familial thrombophilia

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Summary

Few comprehensive data are available on the recurrence rate of venous thrombosis in carriers of thrombophilic defects from thrombophilic families. We prospectively determined the recurrence rate after a first venous thrombotic event in patients with familial thrombophilia due to factor V Leiden or deficiencies of protein C, S or antithrombin. Data were gathered during follow-up on the occurrence of risk situations, anticoagulation treatment and events (e.g., venous thrombosis, hemorrhage). Over a mean follow-up period of 5.6 years, 44 of the 180 (24%) patients with familial thrombophilia who did not use long-term anticoagulation experienced a recurrent venous thromboembolic event (5.0%/year; 95% CI 3.6-6.7) compared with 7 of the 124 patients (6%) on long-term anticoagulation (1.1%/year; 95% CI 0.4-2.2). Spontaneous events occurred less often in patients on long-term anticoagulation (57%) than in patients without long-term anticoagulation (75%). The highest recurrence rate was found among men and women with antithrombin deficiency. Although long-term anticoagulation treatment decreased the incidence of recurrent events with 80%, it also resulted in a risk of major hemorrhage of 0.8% per year. A positive benefit-risk ratio of long-term anticoagulation after a first event is most likely present in patients with familial antithrombin deficiency.

Introduction

A prior history of venous thrombosis is a strong predictor for venous thrombosis, with recurrences in 6-13% of consecutive patients after 1 year and 13-28% after 5 years.[1-5] Patients with a first venous thrombotic event will receive oral anticoagulant treatment for at least 3 months after a deep vein thrombosis and at least 6 months after a pulmonary embolism. Decisions on extending anticoagulant treatment are based on the individual's respective risks of venous thromboembolism recurrence and anticoagulant-related bleeding. Whether long-term continuation of anticoagulant treatment should be considered after a first venous event in carriers of a thrombophilic defect from thrombophilic families is still uncertain, as few comprehensive data are available on whether inherited risk factors increase the risk of recurrence. As was shown by us previously, it might be important to distinguish between unselected patients with a thrombophilic defect and individuals from thrombophilic families, as the latter tend to have a much higher risk at onset of disease due to the concomitant presence of other risk factors within these families.[6,7] To our knowledge, only two studies described the recurrence risk associated with familial deficiencies of protein C, protein S and antithrombin: Pabinger *et al*. found a high recurrence rate of 63% for patients with

familial deficiencies of natural anticoagulant factors[8], and annual incidences calculated on available literature by Van den Belt *et al*. ranged from 13 to 17% for patients with familial antithrombin deficiency and from 14 to 16% for patients with familial protein S deficiency.[9] In the same paper by Van den Belt *et al*., the results of a retrospective cohort study revealed a cumulative incidence of 10% after 1 year and 23% after 5 years for patients with inherited antithrombin-, protein S- or protein C deficiency.[9] For factor V Leiden contradicting results have been published about the risk of recurrent venous thrombosis[10-14], but only one study focused on patients with a positive family history and concluded that the incidence of recurrent venous events depends on the presence of other thrombophilic disorders.[15]

We started the European Prospective Cohort on Thrombophilia (EPCOT) study, a large prospective cohort study of individuals with familial thrombophilia, to obtain reliable risk estimates of the absolute risk of venous thrombosis in families with inherited thrombophilia. The aim of the current analysis was to determine the recurrence risk for participants with a hereditary thrombophilic defect and a history of one venous thromboembolic event before study entry. We focused on selected families with a clear thrombotic tendency, and the results could assist physicians in decisions on testing on thrombophilic abnormalities and choices about long-term anticoagulation after a first thrombosis.

Methods

Participants

The design of the study has been described in detail previously.[16] In brief, all EPCOT participants were included between March 1994 and September 1997. Follow-up was until January 2001. Nine centers from eight European countries (Austria, France, Germany, Great Britain, Italy, The Netherlands, Spain, and Sweden) with expertise on thrombophilia research enrolled registered individuals: both probands (the first of a family identified with thrombophilia) and their registered relatives with inherited thrombophilia. The defects of interest were deficiencies of antithrombin, protein C, protein S or factor V Leiden. Recruitment was before description of the prothrombin G20210A polymorphism. During prospective follow-up, we gathered information on the presence of the prothrombin G20210A polymorphism as a second defect for 207 of the 304 patients (68%) with one venous thromboembolic event at inclusion. Data recorded by the responsible physician or other health professional during follow-up included the occurrence of acquired risk situations (surgery, hospital admission, plaster cast, prolonged bed rest (for more than 2 weeks), traveling (for more than 8 hours), pregnancy, use of female hormones), and anticoagulation treatment and event details (e.g.,

venous thrombosis, hemorrhage, death). All participants in this study gave informed consent.
From all 1626 thrombophilic participants included in the study at baseline, this paper includes only those who had had one objectively confirmed deep venous thrombosis (DVT) or pulmonary embolism (PE)), or a DVT and PE as single event before inclusion in the EPCOT study. Thus, all individuals who were asymptomatic at entry or had suffered more than one thrombosis before study entry were excluded. We also did not include families in which thrombophilia was detected because of screening before hormone prescription or research purposes to stay as close as possible to the real-life situation of an individual from a symptomatic thrombophilia family asking a physician for advice.
We distinguished two treatment groups: thrombophilic patients who did, and those who did not receive long-term prophylactic anticoagulant treatment during prospective follow-up. Long-term anticoagulation was defined as prophylactic treatment for at least one year or throughout the total follow-up period if the follow-up time was less than one year. Thrombophilic individuals who received anticoagulation treatment for more than 1 year after a non-confirmed DVT, PE or superficial thrombophlebitis were also considered to have received long-term anticoagulation. The decision to prescribe long-term treatment was left to the treating physician, and based on various local policies and individual considerations.

Quality assessment of thrombophilia testing and verification of events

All centers performed the various assays according to their local protocol and participated in an external quality assessment scheme for thrombophilia testing (ECAT/UK NEQAS). An adjudication committee, who received all the relevant medical information, scored all reported venous thromboembolic events and hemorrhages independently. Venous thromboembolic events were classified as definite when they were objectively confirmed, i.e. for DVT by ultrasound, impedance plethysmography, venography, or leg symptoms plus a definite PE, and for a PE by angiography, high probability ventilation-perfusion-scan, spiral computerized tomography or autopsy. Superficial venous events were considered definite events when diagnosed by a physician (and not only by the patient). Hemorrhages were classified into severe hemorrhages (requiring hospital admission for emergency diagnosis or treatment), life-threatening hemorrhages (leading to either surgery, irreversible functional damage, or two out of the following three symptoms; severe blood loss, hypotension (systolic blood pressure<90 mm Hg) and anemia (<4mmol/l hemoglobin)), and fatal hemorrhages (rapidly leading to death). Adjudication committee members (FRR, IDW, JF, FEP) agreed on the classification of events in 83 of the 86 (97%) venous thrombotic events and 10 of the 12 (83%) severe hemorrhages experienced by the thrombophilic patients during

prospective follow-up. Only definite events were included in the analysis reported here. A DVT or PE was considered a first event regardless of previous superficial thrombophlebitis and was considered a single event when both DVT and PE were diagnosed within 3 months.
Spontaneous venous thromboembolic events were defined as venous thrombosis without known precipitating risk factors (hospital admission, surgery, immobilization, plaster cast, uninterrupted travels over 8 hours, pregnancy, delivery). Events that had occurred before study entry during the use of female hormones were labeled as unprovoked due to a lack of detailed information.[16] However, during prospective follow-up, we did label events during use of female hormones as provoked.

Analysis and statistics

For all symptomatic patients, we calculated the absolute risk (incidence) of recurrent venous thrombosis during follow-up by dividing the number of events by the total of observation-years (follow-up time). Follow-up time was defined as the time between inclusion and the event of interest, death, or the last date of follow-up (the end of the study or last date before loss-to-follow-up), whichever occurred first. In addition, we calculated the incidence of severe hemorrhage among patients on long-term anticoagulation. We calculated the incidences for the group without long-term anticoagulation on an intention-to-treat basis, thus follow-up time was not corrected for the time individuals did or did not receive short-term treatment. For those on long-term anticoagulation, follow-up time was defined as the time between the start of anticoagulation treatment and the event of interest, death, the last date of anticoagulation treatment or the last date of follow-up, whichever occurred first. Calculation of the 95% confidence intervals was according to a Poisson distribution for the number of events.[17] At study entry, we gathered information from all symptomatic carriers on the year (before study entry) in which their first venous event had occurred. The year of a first event ranged among the symptomatic patients from 1957 to 1995, therefore we calculated the recurrent event-free survival according to the number of observation years instead of the number of follow-up years using the Kurtzke method.[18] This means that subjects who entered the study in 1995 and had their first event in 1985 will only contribute to the survival curve from year 10 on. To determine the benefit of long-term anticoagulation, a hazard ratio as estimation of the relative risk of venous thrombosis was calculated by Cox-regression, with venous thrombosis as the outcome variable and treatment policy (long-term or not) as independent variable. Center, age (as stratum: age<45 and age>=45), and sex were entered in the Cox-regression model to adjust for regional, age and sex effects.

Results

Prospective data were collected on 304 patients with a history of one objectively confirmed DVT or PE before inclusion in the follow-up study. Of these subjects, 124 patients (69 probands and 55 relatives) were classified as being on long-term anticoagulation during prospective follow-up, while 180 patients (139 probands and 41 relatives) were not on long-term prophylactic anticoagulation. The total prospective follow-up time of the 304 patients was 1710 years (mean 5.6; range 1-7 years). During follow-up, twelve patients were lost to follow-up and six patients died as a result of heart disease (n=2), cancer (n=2), septic shock (n=1) and pneumonia (n=1).
Table 1 depicts the main characteristics at inclusion of the patients. Among those not on long-term anticoagulation there were relatively fewer men and more probands than among those who received long-term anticoagulation. The latter can be explained by a low frequency of long-term anticoagulation among probands with factor V Leiden.

Recurrence rates

Of the 180 patients who did not receive long-term anticoagulant treatment during prospective follow-up, 44 (24%) experienced a recurrent thrombotic event (Table 2) compared with 7 of the 124 (6%) patients on long-term anticoagulant treatment (Table 3). Of the patients who did not receive long-term anticoagulant treatment, 4 used aspirin and 2 used heparin (short-term during surgery and pregnancy) at the time of the event. Thirty-three of the 44 events (75%) in those not on long-term anticoagulation occurred spontaneously, and the 11 remaining events occurred after traveling for more than 8 hours (n=5), during pregnancy (n=3), and after surgery (n=3). Of the patients on long-term anticoagulation, 4 of the 7 events (57%) occurred spontaneously: two events occurred during or after pregnancy, and one event occurred after strenuous exercise (karate). Thirteen of the 180 patients (7%) who did not use long-term anticoagulation experienced a superficial thrombophlebitic event during prospective follow-up (i.e. as the only venous recurrent event or before a recurrent DVT or PE) compared with 9 of the 124 patients (7%) on long-term anticoagulation.
The recurrence rate was 5.0% per year (95% CI 3.6-6.7) in patients who were not on long-term anticoagulation and 1.1% per year (95% CI 0.4-2.2) in those on long-term anticoagulation (Tables 2 and 3). Thus, the risk of recurrent events was 80% lower in the patients on long-term anticoagulation treatment (crude relative risk 0.2; 95% CI 0.1-0.4; unchanged when adjusted for center, sex and age at inclusion). In patients who did not receive long-term anticoagulation treatment, the recurrence rate was much higher among men (9.6%/year; 95% CI 6.3-13.9) than among women (2.8%/year; 95% CI

Table 1 *General characteristics of the thrombophilic subjects with 1 confirmed DVT or PE at study entry*

	Patients (n=304)	
	No long-term anticoagulation	Long-term anticoagulation
All, n	180	124
Men, n (%)	63 (35)	59 (48)
Probands, n (%)	139 (77)	69 (56)
Age at inclusion, mean years (range)	39 (14-78)	41 (20-72)
Age at 1st event, mean years (range)	32 (11-70)	33 (13-71)
Type of thrombophilia		
Protein C deficiency, n	37	32
Protein S deficiency, n	25	30
Antithrombin deficiency, n	11	26
Factor V Leiden, n	79 (12 AA)	13 (4 AA)
Combined defects, n	28	23
PC-PS	0	1
PC-FVL	6	7
PC-PT	3	4
PC-FVL-PT	2	0
PS-FVL	4	4
PS-PT	2	2
AT-FVL	2	3
AT-PT	2	1
FVL-PT	7	1[#]
Risk factors		
BMI, mean kg/m^2 (range)	25 (17-47)	25 (17-34)
Cancer ever, %	2	0
Arterial disease, %	3	9
Thrombosis history		
DVT, n (%)	133 (74)	79 (64)
PE, n (%)*	47 (26)	45 (36)
Spontaneous DVT or PE, n (%)**	105 (58)	62 (50)
History of STPs, n (%)	37 (21)	40 (32)
Time between 1st event and study entry, mean years (range)	6.4 (0-38)	7.3 (0-33)

Abbreviations: PC=protein C deficiency, PS=protein S deficiency, AT=antithrombin deficiency, FVL= factor V Leiden, AA=homozygotes for factor V Leiden, PT=prothrombin G20210A, BMI= body mass index, DVT=deep venous thrombosis, PE=pulmonary embolism, STP=superficial thrombophlebitis. *A DVT and PE were experienced at the same time by 29 patients without and 29 patients with long-term anticoagulation. **Defined at entry as events in the absence of the following risk situations: surgery, cancer, hospitalization, plaster cast, immobilisation, pregnancy, and travelling. [#]One was homozygous for FVL.

Table 2 *Incidence rates (%/year) of a recurrent DVT or PE in those who did not receive long-term anticoagulant treatment during prospective follow-up*

Patients	n	Time between 1st event and study entry, mean years (range)	DVT/PE, n	Person years, n	Incidence rate, %/year (95% CI)	Incidence rate men, %/year (95% CI)	Incidence rate women, %/year (95% CI)
All	180	6.4 (0-38)	44*	881.7	5.0 (3.6-6.7)	9.6 (6.3-13.9)	2.8 (1.7-4.5)
PC deficiency	37	6.3 (0-27)	10	195.0	5.1 (2.5-9.4)	10.8 (4.0-23.4)	2.9 (0.8-7.4)
PS deficiency	25	5.3 (0-16)	8	122.4	6.5 (2.8-11.8)	10.5 (3.9-22.9)	3.1 (0.4-11.0)
AT deficiency	11	9.3 (1-24)	6	57.4	10.5 (3.8-22.8)	11.6 (2.4-33.9)	9.5 (2.0-27.8)
Factor V Leiden	79	5.3 (0-38)	13 (1 AA)	366.3	3.5 (1.9-6.1)	7.2 (2.9-14.9)	2.2 (0.8-4.8)
Combined defects	28	9.4 (1-30)	7**	140.6	5.0 (2.0-10.3)	10.7 (3.5-24.9)	2.1 (0.3-7.7)

Abbreviations: DVT=deep venous thrombosis, PE=pulmonary embolism, PC=protein C, PS=protein S, AT=antithrombin, AA=homozygotes for factor V Leiden, CI=confidence interval. *Thirty-four experienced a DVT, 6 a PE and 4 a DVT and PE concurrently. **Three with protein C deficiency and factor V Leiden, 1 with protein C deficiency and the prothrombin 20210A variant, 1 with factor V Leiden and the prothrombin 20210A variant, 1 with protein C deficiency, factor V Leiden and the prothrombin 20210A variant and 1 with antithrombin deficiency and the prothrombin 20210A variant.

1.7-4.5) (Table 2) with a relative risk of 3.0 (95% CI 1.6-5.6), which remained unchanged when adjusted for age (relative risk 3.1; 95% CI 1.6-5.9) or when we restricted the analysis to those with a first spontaneous venous thrombotic event (relative risk 3.1; 95% CI 1.4-7.1). Amongst the thrombophilic women who were not on long-term anticoagulation, measures to reduce the risk of recurrence associated with pregnancy or use of female hormones were frequently employed: 25 of the 29 (86%) women who were pregnant during prospective follow-up received thromboprophylaxis during pregnancy or puerperium, and only 13 of the 100 (13%) women between the age of 15 and 50 used oral contraceptives, of whom 9 women used oral contraceptives which contained no estrogen. Amongst the thrombophilic women who were on long-term anticoagulation 10 of the 50 (20%) women between the age of 15 and 50 used oral contraceptives, of whom 9 women used estrogen-containing oral contraceptives.

The lowest recurrence rates, although the confidence intervals were wide, were found in patients with factor V Leiden (3.5%/year without and 0.0%/year with long-term treatment), and the highest recurrence rates in patients with antithrombin deficiency (10.5%/year without and 2.7%/year with long-term treatment) (Tables 2 and 3). However, when we calculated the incidences for men and women separately, we found among those not on long-term anticoagulation high and similar recurrence rates for men with protein C, protein S or antithrombin deficiency and men with multiple defects (10.5-11.6%/year; Table 2). For men with factor V Leiden we found a lower rate (7.2%/year; Table 2). For women, except for those with antithrombin deficiency (9.5%/year), the recurrence rates were much lower (2.1-3.1%/year; Table 2). Besides a difference in the recurrence rate among men and

Table 3 *Incidence rates (%/year) of a recurrent DVT and/or PE in patients on long-term anticoagulant treatment during prospective follow-up*

	n	Time between 1st event and study entry, mean years (range)	DVT/PE, n	Person years (during treatment only), n	Incidence rate, %/year (95% CI)
All	124	7.5 (0-36)	7*	652.0	1.1 (0.4-2.2)
PC deficiency	32	6.7 (0-29)	1	172.1	0.6 (0.0-3.2)
PS deficiency	30	6.2 (0-20)	1	172.8	0.6 (0.0-3.2)
AT deficiency	26#	6.7 (0-26)	4	146.9	2.7 (0.7-7.0)
Factor V Leiden	13	7.3 (1-24)	0	43.4	0.0 (0.0-8.5)
Combined defects	23	11.2 (0-36)	1**	116.8	0.9 (0.0-4.8)

Abbreviations: DVT=deep venous thrombosis, PE=pulmonary embolism, PC=protein C, PS=protein S, AT=antithrombin, CI=confidence interval, N/a= not applicable. *Five patients experienced a DVT, 1 a PE and 1 a cerebral venous event. **This individual was protein S deficient and factor V Leiden carrier. #One individual received antithrombin concentrate as anticoagulation therapy.

women who received no long-term anticoagulation, we found, although confidence intervals were wide, a higher recurrence rate in the probands, in the patients with a spontaneous first past event and in those with a concurrent DVT and PE as first event (Table 4). In patients on long-term anticoagulation, recurrence rates were independent of sex (recurrence rates of 1.0%/year for men and 1.2%/year for women), and the etiology of the first event (spontaneous or not) (Table 4). The influence of risk situations on the risk of venous thrombosis was difficult to determine as short-term prophylaxis was prescribed frequently in patients who were not on long-term anticoagulation: in 6 of the 11 individuals wearing a plaster cast, in 7 of the 83 patients who traveled and in 29 of the 71 patients undergoing surgery or hospitalization.

Table 4 *Incidence rates (% per year) in subgroups*

	Incidence, % per year (95% CI)	
	No long-term anticoagulation (N=180)	Long-term anticoagulation* (N=124)
Probands	5.6 (4.0-7.7)	1.4 (0.5-3.3)
Relatives	3.1 (1.3-6.5)	0.7 (0.1-2.4)
History spontaneous events	5.7 (3.8-8.2)	1.2 (0.3-3.2)
No history spontaneous events	4.0 (2.2-6.6)	0.9 (0.2-2.7)
History of concurrent DVT/PE	8.5 (4.4-14.8)	1.1 (0.1-4.1)
History of DVT or PE only	4.3 (3.0-6.1)	1.0 (0.3-2.4)

Abbreviations: CI=confidence interval, DVT=deep venous thrombosis, PE=pulmonary embolism.
* Incidence was in percentage per year on thromboprophylaxis

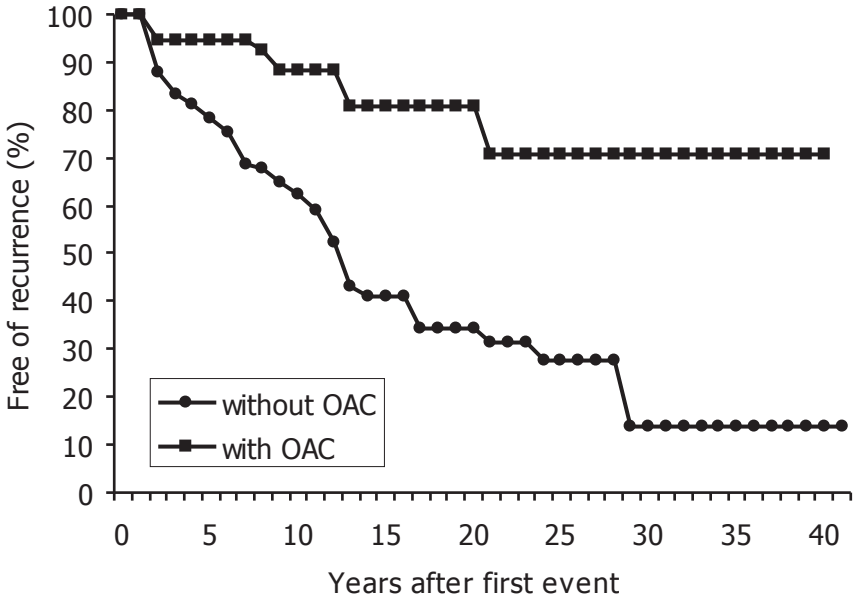
Figure 1 shows the recurrence-free survival curve according to the number of observation years. For the subjects who were not on long-term anticoagulation, five individuals were included in the same year as their first event occurred, and 28 individuals entered the study in 1994 or 1995 and had experienced their first event in, respectively, 1993 or 1994. After 2 years of observation, these 5 and 28 individuals were followed prospectively for, respectively, 2 years and 1 year, and 12% of these 33 individuals had experienced a recurrent event (Figure 1). After 5 years of observation, an additional 10% of the subjects had experienced an event (Figure 1). Recurrence-free survival was higher for subjects who were on long-term anticoagulation: after 2 years of observation, 7 and 11 individuals were followed prospectively for, respectively, 2 years and 1 year, and 6% of these

18 individuals had experienced a recurrent event (Figure 1). After 5 years of observation, no additional subjects had experienced an event (Figure 1).

Risk from prophylactic anticoagulation

Two women who did not use long-term anticoagulation experienced a severe hemorrhage during delivery while receiving short-term heparin treatment to prevent thrombosis during pregnancy. Five patients experienced a severe hemorrhage during the use of long-term anticoagulation (3 gastro-intestinal hemorrhages and 2 post-operative bleeding episodes after a hysterectomy or a thrombectomy) requiring hospital admission for an emergency diagnosis or for treatment. One additional patient experienced a post-operative hemorrhage after the removal of a melanoma, but had stopped oral anticoagulation two weeks before during treatment with penicillin for erysipelas. The incidence of severe hemorrhages associated with the use of oral anticoagulation was 0.8% per year (95% CI 0.3-1.8; 5 events on 638 years of anticoagulation).

Figure 1 *Percentage of individuals who were free of a recurrent venous event according to the number of years after a first event (calculated using the Kurtzke method)*



Discussion

The recurrence rate of venous thrombosis after a first DVT or PE was 5.0% per year (95% CI 3.6-6.7) in the 180 patients who did not receive long-term anticoagulation during prospective follow-up. In these patients, the recurrence rate of venous thrombosis was much higher in men than in women with a relative risk, adjusted for age, of 3.1 (95% CI 1.6-5.9), which is in accordance with a recent paper by Kyrle *et al*. who reported a relative risk of recurrence of 3.6 (95% CI 2.3-5.5) for men compared with women.[19] However, it should be noted that measures to reduce the risk of recurrence associated with pregnancy or use of female hormones were frequently employed. The incidence was also higher for patients in whom the first event had been a spontaneous event, as previously reported by others.[20-22] In addition, we found a higher recurrence rate in patients who had experienced a DVT and PE concurrently as a first event than in those with a DVT or PE as first event. For factor V Leiden carriers the recurrence rate (3.5% per year) was similar to the incidences found in several prospective and retrospective studies on unselected patients with factor V Leiden of about 5% per year.[10-13] The risk of a recurrent event in unselected patients with a first thrombotic event is reported to be highest in the first year after a first event (6-13% after 1 year compared with 13-28% after 5 years).[1-5] However, as most symptomatic individuals in the present study did not enter the study in the same year as they had experienced their first event, our rates are less comparable to the rates found in other studies. When we took the year of a first event into account, we found that 12% of the subjects who did not receive long-term anticoagulation had experienced a recurrent event after 2 years. After 5 years of observation, an additional 10% had experienced a recurrent event. A discrepancy with earlier found rates[1-5] could be explained by the fact that we only had information on the year of a first event and not the exact date, which might lead to under- or overestimation of the years between a first event and inclusion.

The recurrence rate was 80% lower in the patients using long-term anticoagulation treatment with an incidence of recurrent events of 1.1% per year (95% CI 0.4-2.2). De Stefano *et al*. found a low annual incidence of 1.4% for individuals with antithrombin-, protein S- or protein C deficiency in a retrospective study in which most individuals received life-long prophylaxis.[23] Their finding is similar to the recurrence rate found in the present study for patients with protein C-, protein S- or antithrombin deficiency receiving long-term anticoagulation (1.2%/year; 95% CI 0.4-2.7), although the recurrence rate for antithrombin deficiency remained high (2.7% per year). The reduction of the recurrence rate with 80% came with an increase in the risk of severe hemorrhages during use of long-term anticoagulation of 0.8% per year (95%

CI 0.3-1.8), which is similar to the incidence of major hemorrhages of 1-3% found in other studies.[24,25]
The strength of our study is its size due to the collaboration of several European thrombosis centers, which made the restriction to patients with one venous thrombotic event possible. However, the present study was an observational study, and it is possible that those on long-term anticoagulation were those with a high recurrence risk and that those not on long-term anticoagulation were those with a lower recurrence risk or a past history of (severe) hemorrhage: randomized studies will be required to demonstrate whether the difference in recurrence and bleeding risk brought about by long-term treatment is indeed as we have found, although the low prevalence of some of the thrombophilic disorders may render this infeasible. Likewise, the availability of anticoagulants with a better risk-benefit profile could change the balance. In addition, this study focused on subjects already tested for inherited thrombophilia, and due to preventive strategies their risk of recurrence may have been lower than in untested patients; hence, we cannot fully infer the effects of testing for thrombophilic defects. We did not include individuals with a non-objectively confirmed recurrent venous event only; however, these were few (n=4).
Generally, the recurrence rate in patients with familial thrombophilia was similar to the recurrence rate found in consecutive patients in the absence of long-term anticoagulation treatment. However, we found a much higher recurrence rate in men, and a high recurrence rate for patients, men and women, with antithrombin deficiency (over 10%/year). In the patients who received long-term anticoagulation, the recurrence rate was 80% lower, at an increased risk of severe hemorrhages. A positive benefit-risk ratio of long-term anticoagulation after a first event is most likely present in patients with antithrombin deficiency, who still showed a recurrence rate of 2.7% on long-term anticoagulation.

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Chapter I.4

Hereditary thrombophilia and fetal loss:

a prospective follow-up study

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Summary

As the placental vessels are dependent on the normal balance of procoagulant and anticoagulant mechanisms, inherited thrombophilia may be associated with fetal loss. We performed a prospective study to investigate the relation between inherited thrombophilia and fetal loss, and the influence of thromboprophylaxis on pregnancy outcome. Women were enrolled in the European Prospective Cohort on Thrombophilia (EPCOT). These included women with factor V Leiden or a deficiency of antithrombin, protein C or protein S. Controls were partners or acquaintances of thrombophilic individuals. A total of 191 women (131 with thrombophilia, 60 controls) had a pregnancy outcome during prospective follow-up. Risk of fetal loss and effect of thromboprophylaxis were estimated by frequency calculation and Cox-regression modelling. The risk of fetal loss appeared slightly increased in women with thrombophilia without a previous history of fetal loss who did not use any anticoagulants during pregnancy (7/39 versus 7/51; relative risk 1.4; 95% CI 0.4-4.7). Per type of defect the relative risk varied only minimally from 1.4 for factor V Leiden to 1.6 for antithrombin deficiency compared to control women. Prophylactic anticoagulant treatment during pregnancy in 83 women with thrombophilia differed greatly in type, dose and duration, precluding solid conclusions on the effect of thromboprophylaxis on fetal loss. No clear benefit of anticoagulant prophylaxis was apparent. Women with thrombophilia appear to have an increased risk of fetal loss, although the likelihood of a positive outcome is high in both women with thrombophilia and in controls.

Introduction

Thrombotic risk is increased in normal pregnancy, which may be the result of an evolutionary advantage associated with reduced blood loss after delivery and placenta separation. This risk is further enhanced in women with congenital or acquired thrombophilia.[1] In addition it has been shown that postpartum blood loss is reduced in women with factor V Leiden.[2,3] As an adequate placental circulation is dependent on the normal balance of procoagulant and anticoagulant mechanisms, inherited thrombophilia may be associated with fetal loss.

In a retrospective analysis[4] of women participating in the European Prospective Cohort on Thrombophilia (EPCOT) study, we observed a slightly increased risk of fetal loss in women with hereditary thrombophilia (168/571 versus 93/395; odds ratio 1.35; 95% confidence interval (CI) 1.10-1.82). Several other studies have confirmed an association between thrombophilia and fetal loss.[5-18] Subsequent to our retrospective baseline analysis[4], we followed the women in the EPCOT cohort prospectively to investigate the relation between fetal

loss and inherited thrombophilia. The prospective study design allowed us to study women in a well-defined and homogeneous time frame with homogeneous general conditions, as well as to reduce recall bias. To prevent venous thromboembolism during pregnancy, pregnant women with inherited thrombophilia often receive thromboprophylaxis, such as heparin, oral anticoagulants or aspirin. This policy is a matter of controversy, and therefore applied in some centers, but not in all. As thromboprophylaxis could prevent fetal loss by preventing thrombosis in fetal placental vessels, we evaluated the effect of anticoagulants during pregnancy on pregnancy outcome.

Subjects and methods

Subjects

The women described in this study were enrolled in the EPCOT study between March 1994 and September 1997 and subsequently followed prospectively. The primary aim of the EPCOT-study was to establish the risk of thrombosis in individuals with inherited thrombophilia. Nine centers in 8 countries (Austria, France, Germany, Great Britain, Italy, The Netherlands, Spain, and Sweden) participated and followed all consenting individuals with a deficiency of natural coagulation inhibitors (antithrombin, protein C, protein S), resistance to activated protein C due to factor V Leiden, or a combination of these defects. As controls, partners or, if there were none, friends or acquaintances of the thrombophilic participants were included. Data were collected at baseline, and annually at follow-up by questionnaire, telephone or personal interview until January 2001. For follow-up data, information on the occurrence of risk situations or events was screened by questionnaire or telephone, and, when reported, further information was obtained from a physician. The data recorded at baseline included general demographic information, history with regard to thrombosis, current medication, obstetric history, family information and (only for thrombophilic individuals) details on type and subtype of thrombophilia. The data recorded at follow-up included the number of births, pregnancies and miscarriages, current medication and occurrence of risk situations for thrombosis. While there were no exclusion criteria for thrombophilic individuals, controls were excluded when they were blood relatives of an individual with an inherited thrombotic defect or were known to have heritable thrombophilia.

For assessing the risk of fetal loss, we selected from the participating women those aged between 13 and 45, with at least one pregnancy ending in livebirth or fetal loss during prospective follow-up. We excluded women with only elective pregnancy terminations. Partners of men in the cohort or female acquaintances of thrombophilic individuals were included as controls.

Analysis

Only the first pregnancy during prospective follow-up (regardless of previous pregnancies reported before inclusion) was considered for the analysis. Risk was expressed as the frequency of fetal loss, i.e., the proportion of all first pregnancies ending in fetal loss. Fetal loss was defined as any loss during pregnancy independent of time of occurrence.
In the analysis aimed at assessing the risk of fetal loss associated with thrombophilia, we excluded women who used thromboprophylaxis during their first pregnancy during prospective follow-up. The effect of anticoagulants on pregnancy outcome was analyzed by comparing the risk of fetal loss between those with anticoagulants (heparin, oral anticoagulants) during the first pregnancy and those without thromboprophylaxis during the first pregnancy since study entry. Both calculations were performed per pregnant woman (i.e., not per pregnancy) by Cox-regression analysis with fetal loss as the outcome (dependent) variable and presence of thrombophilia or use of prophylaxis as covariate (independent variable). Follow-up time was the number of weeks of pregnancy. To adjust for regional effects and the influence of the number of previous pregnancies, center (as stratum) and the total number of pregnancies (as independent variable) were added in the Cox regression model. We also considered the effect of a history of fetal loss or venous thrombosis and the influence of gestation at age 35 or older.

Results

A total of 1573 women were enrolled in the cohort (988 with thrombophilia, 585 controls), of whom 191 completed at least one pregnancy during prospective follow-up (131 with thrombophilia, 60 controls). Of the controls, 37 (62%) were female partners and 23 (38%) were friends of participants with thrombophilia. Among the 131 thrombophilic women, 83 (63%) used thromboprophylaxis during the first pregnancy in prospective follow-up. None of the controls used thromboprophylaxis during pregnancy. The main characteristics are shown in Table 1. Most women had their first fetal loss during prospective follow-up. A history of venous thrombosis was present predominantly among women with thrombophilia who used thromboprophylaxis during the first pregnancy since study entry.

Fetal loss

A total of 108 women (48 with thrombophilia, 60 controls) did not use thromboprophylaxis during the first pregnancy since study entry. Fetal loss occurred among 10 of the 48 women with thrombophilia (21%), and among 10 of the 60 control women (17%). The relative risk for fetal loss associated with thrombophilia was 1.4 (95% CI 0.5-3.8); adjusted for center and the

Table 1 *Inclusion characteristics of women pregnant during prospective follow-up*

	Women with thrombophilia (n=131)		
	First pregnancy after study entry without thromboprophylaxis (n=48)	First pregnancy after study entry with thromboprophylaxis (n=83)	Controls (n=60)
Age at inclusion, mean (range)	28.7 (19-41)	29.1 (16-39)	28.7 (13-38)
History of pregnancies, n (%)*	30 (63%)	48 (58%)	34 (57%)
History of fetal loss, n (%)#	9 (19%)	12 (14%)	9 (15%)
History of VT, n (%)	9 (19%)	43 (52%)	0 (0%)

Abbreviation: VT=Venous thrombosis *Range of number of pregnancies was 1-7 in women with thrombophilia and 1-5 in controls. #All women had 1 previous fetal loss.

number of pregnancies. In both thrombophilic women and controls, the risk of fetal loss was higher if they had a history of fetal loss (3/9 (33%) in both women with thrombophilia and controls) or when women were ≥35 years old or older at gestation (4/9 (44%) in women with thrombophilia, 3/9 (33%) in controls) (Table 2). A history of venous thrombosis (only present in women with thrombophilia) did not affect the risk of fetal loss. Further adjustment for a history of fetal loss and age at gestation (<35 vs ≥35) resulted in a relative risk of 1.7 (95% CI 0.6-4.6) for thrombophilic women versus controls. Exclusion of women with a history of fetal loss resulted in a relative risk of 1.4 (95% CI 0.4-4.7) for thrombophilia carriers versus pregnant control women, adjusted for age at pregnancy, regional effects and number of total pregnancies. Per type of defect, the relative risk, adjusted for age at gestation and number of previous pregnancies, varied little: 1.4 (95% CI 0.3-5.5) for the factor V Leiden mutation, 1.5 (95% CI 0.2-12.9) for protein S deficiency, 1.5 (95% CI 0.3-7.9) for protein C deficiency and 1.6 (0.3-14.0) for antithrombin deficiency. Of all thrombophilic women and controls with no previous pregnancies before prospective follow-up, 2 of the 18 (11%) thrombophilic women and 5 of the 26 (19%) controls experienced fetal loss. The mean number of weeks at which fetal loss took place was week 9 (range 4-18) in women with thrombophilia and week 9 (range 4-17) in controls. Eight women with thrombophilia (80%) and nine controls (90%) experienced a first trimester fetal loss (between week 0 and 12) with a relative risk for first-trimester fetal losses of 1.6 (95% CI 0.5-4.7) adjusted for age at pregnancy, regional effects and number of total pregnancies. The frequency of caesarian sections was the same for women with thrombophilia (21%) and control women (20%).

For 91 of the 131 pregnant women with thrombophilia, we had information on genotyping for the prothrombin G20210A mutation. None of the 12 women

with this mutation (11 heterozygous, 1 homozygous) experienced fetal loss. So, among a total of 14 women with combined defects, no pregnancy loss was observed.
Most of the controls were partners of men with a thrombotic defect. We assessed the effect of paternal thrombophilia on pregnancy outcome, and found none: the relative risk of fetal loss for partner controls versus friend controls was 0.4 (95% CI 0.1-2.2), adjusted for center, age at gestation, number of pregnancies and history of fetal loss.

Table 2 *Fetal loss in women without thromboprophylaxis (heparin, oral anticoagulants, aspirin) during pregnancy*

	Women with thrombophilia						Controls
	Single defects				Combined defects	Total	
	PC	PS	AT	FVL*			
Pregnant, n	13	7	5	21	2#	48	60
Fetal loss, n	4	2	1	3	0	10 (21%)	10 (17%)
History of fetal loss, n	2	1	0	5	1	9	9
Fetal loss , n	2	1	0	0	0	3 (33%)	3 (33%)
Age at pregnancy ≥ 35, n	1	2	1	4	1	9	9
Fetal loss, n	1	1	0	2	0	4 (44%)	3 (33%)

Abbreviations: PC=Protein C deficiency, PS=protein S deficiency, AT=antithrombin deficiency, FVL= factor V Leiden mutation. *Only heterozygotes. #Both had protein S deficiency and the factor V Leiden mutation.

Effect of using anticoagulants

A total of 83 women with thrombophilia received thromboprophylaxis, i.e. heparin (n=77), oral anticoagulants (n=5) or aspirin (n=1), at some point during the first pregnancy in prospective follow-up. The frequency of thromboprophylaxis varied among centers between 31% and 93% of all pregnant women with thrombophilia. The percentage of women receiving thromboprophylaxis was high for women with antithrombin deficiency or combined defects, respectively 82% and 83%, compared with 61% of the women with protein C deficiency, 70% of the women with protein S deficiency and 42% of the women with the factor V Leiden mutation. The indication for thromboprophylactic treatment during pregnancy was known for 71 women (86%), of whom only 20 women (28%) received thromboprophylaxis to prevent fetal loss. The type, dose and duration of anticoagulant treatment therefore ranged greatly among the women with thrombophilia. Among 21

women with thrombophilia who used heparin or oral anticoagulants before week 5 of the pregnancy until the end of the pregnancy, 5 (24%) experienced fetal loss with an unadjusted relative risk of fetal loss associated with thromboprophylaxis of 1.1 (95% CI 0.4-3.3), and a relative risk adjusted for center, total number of pregnancies, history of fetal loss and age at gestation of 0.7 (95% CI 0.2-3.2). The mean pregnancy duration until miscarriage in these women was 13 weeks (range 6-29).

Discussion

Earlier retrospective data in the EPCOT study showed that the risk of fetal loss was increased in women with thrombophilia (168/571 versus 93/395; odds ratio 1.35; 95% CI 1.01-1.82).[4] We found a similar risk during this prospective follow-up study of 1.4 (95% CI 0.4-4.7) for thrombophilic women without a previous history of fetal loss and who did not use any anticoagulants during pregnancy versus controls, adjusted for age, number of previous pregnancies and regional effects. Even though the present study in itself could not exclude the absence of an increased risk with a 95% confidence interval from 0.4 to 4.7, this statistical uncertainty is likely to be the result of the smaller number of pregnancies compared with the retrospective analysis and the results of both analyses are in agreement. A major difference between the prospective and retrospective analysis is that the odds ratio in the retrospective analysis was higher for stillbirth than for miscarriage, whereas none of the women experienced a stillbirth in the prospective analysis. This difference is probably the result of the lower number of individuals followed in the prospective study; 4% of the thrombophilic women experienced a stillbirth in the retrospective study and 27% a miscarriage, so if we take these percentages (which are based on a longer follow-up period), we would expect 2 stillbirths and 13 miscarriages in the 48 women without use of anticoagulants during prospective follow-up.

Per defect, we found similar risks of fetal loss: 1.4 (95% CI 0.3-5.5) for the factor V Leiden mutation, 1.5 (95% CI 0.2-12.9) for protein S deficiency, 1.5 (95% CI 0.3-7.9) for protein C deficiency and 1.6 (0.3-14.0) for antithrombin deficiency. Previously, we found similar odds ratios for miscarriage per type of defect: 0.9 (95% CI 0.5-1.5) for the factor V Leiden mutation, 1.2 (95% CI 0.7-1.9) for protein S deficiency, 1.4 (95% CI 0.9-2.2) for protein C deficiency and 1.7 (95% CI 1.0-2.8) for antithrombin deficiency.[4]

Earlier retrospective studies reported similar[19,20] or higher[10,15] risks of fetal loss in women with a deficiency of antithrombin, protein S or protein C. For factor V Leiden, several previous reports showed an association with late fetal loss (2nd and 3rd trimester)[5,6,8-11,16-18], while reports were contradictory on the association with early fetal loss (1st trimester).[5,7,9,12-14,16-18,21-24] Discrepancies

between studies could be explained by their design: many previous reports were based on case-control studies targeting recurrent miscarriage without a comparison with women in the general population, which precludes risk calculation. According to Bick *et al*, 55 to 62% of recurrent miscarriages are caused by blood coagulation protein or platelet defects, whereas about 90% of first time miscarriages are caused by chromosomal defects.[25] In our study most women (39/48 (81%); Table 1) had their first fetal loss during prospective follow-up. It may well be that thrombophilia plays a role in a small selected group of women with recurrent fetal loss, who are likely to have other, some as yet unknown, defects.

We found no positive effect of thromboprophylaxis on the outcome of a pregnancy in 21 women with thrombophilia who used heparin or oral anticoagulants before week 5 of the pregnancy until the end of the pregnancy (relative risk 0.7; 95% CI 0.2-3.2). However, this group is rather small to draw solid conclusions from and treatment in this group differed per person in type and dose as most women received thromboprophylactic treatment during pregnancy to prevent venous thrombosis. However, since the risk of fetal loss without thromboprophylaxis was only mildly increased, a strong beneficial effect could not be expected in this study. In women with a severe thrombotic tendency and recurrent fetal loss, thromboprophylaxis may offer more benefit, although currently only non-conclusive information is available on thromboprophylaxis for pregnant women with thrombophilia and a history of obstetric complications.[9,26-29]

In conclusion, although the risk of fetal loss is increased in women with thrombophilia, the overall likelihood of a positive outcome is high. Further research regarding prophylactic treatment (heparin, oral anticoagulants or newly developed anticoagulants) should be performed, targetting high-risk women, weighing risks and benefit. At the moment, thrombophylactic treatment does not seem indicated in women without a history of fetal loss for prevention of fetal loss only, while it may be a worthwhile option in women with a severe thrombotic tendency and recurrent fetal loss.

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Chapter I.5

No effect of the prothrombin G20210A mutation on protein C activation in a large kindred with type I protein C deficiency

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Abstract

Previously, we observed a positive association of prothrombin concentrations with thrombin generation (fragment 1+2) and thrombin activity (fibrinopeptide A), but no association with protein C activation peptide levels. We further evaluated a potential beneficial effect of increased prothrombin concentrations on activated protein C generation by assessing the plasma concentration of activated protein C in complex with protein C inhibitor (APC-PCI). Blood samples were used from 195 family members of a large French-Canadian kindred with type I protein C deficiency due to a 3363C insertion in the protein C gene. We utilized a new and highly sensitive assay for measuring the concentration of APC-PCI complex as a measure of the level of activation of protein C. Means of the plasma concentrations of APC-PCI complex were compared among carriers and non-carriers of the prothrombin G20210A mutation. Protein C activity levels were positively associated with APC-PCI complex plasma concentrations; however, APC-PCI complex levels were not different for carriers of the prothrombin G20210A mutation than for non-carriers. Thus, carriers of the prothrombin G20210A mutation do not have increased protein C activation despite the increased thrombin generation resulting from the higher prothrombin concentrations associated with the G20210A mutation.

Introduction

Protein C is a vitamin K dependent (molecular weight 62 000) zymogen for a serine protease that downregulates the hemostatic system through the proteolytic inactivation of factors Va and VIIIa. Protein C deficiency was first associated with thrombophilia in 1981.[1] Heterozygous deficiency of protein C has a prevalence of 1 in 200 in the general population.[2] The incidence of symptomatic disease in penetrant families is considerably higher than in the general population.[3-6] The apparent variable penetrance of thrombotic disease among families with protein C deficiency has been attributed to the co-segregation of additional risk factors. Two likely candidates are the highly prevalent risk factors for thromboembolic disease, factor V Leiden and the prothrombin G20210A polymorphism.[7,8]

Since 1985, we have studied a large kindred of French-Canadian descent with an 8-fold increased risk of venous thrombosis and early onset of disease, associated with a 3363C insertion mutation in the protein C gene.[3] Segregation analysis suggested that the increased risk of thrombosis found in this kindred resulted from the interaction between the protein C 3363C insertion and another unknown genetic defect.[9] Currently, we have identified 787 family members, of whom 450 have been tested for the protein C 3363C

insertion. Factor V Leiden was found in only 4 individuals, and the G20210A prothrombin polymorphism was found in 13%. We found, however, no association between the G20210A prothrombin polymorphism and increased thromboembolic disease, despite the unusually high prevalence in this kindred, including a number of individuals with both protein C deficiency and the G20210A polymorphism.[10]

Since increased thrombin generation has been associated with a higher prothrombin concentration *in vivo*[11,12], we postulated a potential beneficial effect of increased thrombomodulin mediated activated protein C-generation in carriers of the prothrombin G20210A mutation. This hypothesis was not supported in a small preliminary study in which we correlated plasma concentrations of the protein C activation peptide, prothrombin fragment 1+2 and fibrinopeptide A with prothrombin concentration. We observed a positive association of prothrombin concentrations with thrombin generation (fragment 1+2) and thrombin activity (fibrinopeptide A), but no association with protein C activation peptide levels.[11] In the present study we have further evaluated this hypothesis in a larger sample of the family, by assessing the plasma concentration of the complex of activated protein C combined with protein C inhibitor (APC-PCI). Protein C inhibitor (PCI) is a molecular weight 57 000 serine protease inhibitor with a plasma concentration of 90 nM.[13,14] Plasma concentrations of APC-PCI in part reflect the degree of activation of the protein C system. Previously described assays for APC-PCI have not been sensitive enough to accurately measure the full range of concentrations of the complex in healthy individuals. In this study we have utilized a new and highly sensitive assay for measuring the concentration of APC-PCI complex as a measure of the level of activation of protein C.[15]

Methods

Participants

Blood samples were collected from 201 family members of a large French-Canadian kindred with type I protein C deficiency, including spouses of family members who have children. All samples were collected in 2002 into sodium citrate pH 4.3 Stabilyte tubes (Biopool, Umeå, Sweden). The ascertainment and evaluation of the family members was previously described.[3] All subjects completed questionnaires regarding general demographic information, current health status, current medication, obstetric history, and personal history with regard to events (venous as well as arterial thrombosis and hemorrhages) and risk factors for venous thrombosis (i.e. surgeries, hospital admissions, bed rest, plaster cast). Completed forms were stored with only the patient identifier codes to protect patient confidentiality. All participating subjects gave informed consent. This study was approved by the Human Experimentation

Committee of the University of Vermont College of Medicine.
Six individuals using oral anticoagulants at the time of the blood draw had levels of APC-PCI complex ranging from 0.01 to 0.03 µg/L. These individuals were excluded from all calculations.

Laboratory methods

We measured protein C activity levels by performing a clot-based functional assay using a kit provided by Diagnostica Stago (Parsippany, NJ, USA).[3,16] The inter-assay coefficient of variation (CV) of this assay was 5.5%. The presence of the 3363C insertion in the protein C gene was determined by amplification of genomic DNA using a mutagenic oligonucleotide primer that in concert with the inserted C mutation creates a *Bg1*I cleavage site. The product was digested with *Bg1*I and analyzed on a 2% agarose gel.[17] The prothrombin G20210A allele was detected by amplification of genomic DNA with a mutagenic primer resulting in a *Hind*III cleavage site when the A-allele was present.[18]
Concentrations of the APC-PCI complex were measured by a previously described assay.[15] Samples were incubated with monoclonal biotinylated capture antibody M36 which recognizes a conformation dependent neo-epitope in APC-PCI complexes.[15,19] The mean level of APC-PCI complexes for a reference group, consisting of Swedish healthy individuals (n=80; mean age 42 years; 20 men and 60 women), was 0.13 µg/L (range 0.07-0.26).[20] The functional detection limit (intra-assay CV<20%) in Stabilyte plasma is 32 ng/L (unpublished data).

Statistical methods

SPSS was used to calculate the mean and 95% confidence intervals (CIs) (mean ±1.96 x standard error) of the levels of APC-PCI complex. Correlation analysis for levels of APC-PCI complex and protein C activity was performed by calculating Pearson's correlation coefficient, and its non-parametric equivalent the Spearman's rank correlation coefficient. Pearson correlation coefficients were similar to the Spearman's rank correlation coefficients, but because the data were not normally distributed, only the latter are presented. For each Spearman's rank correlation coefficient we calculated the 95% CI.[21] Correlations were calculated without accounting for the family structure. However, the heritability of APC-PCI and protein C were both low enough to perform analyses that do not account for family structure.[22]

Results

APC-PCI levels and information on carriership of the prothrombin G20210A mutation were available for 55 family members with the protein C 3363C insertion and 140 family members without this mutation (35 were spouses). Of these 195 individuals, 83 were men (43%), 19 (10%) had experienced a venous thrombosis in the past, and the prothrombin mutation was present in 24 of the family members (12%). The mean age at the blood draw was 41 years (range 10-78 years).

Table 1 shows that family members with the protein C mutation had lower plasma concentrations of APC-PCI complex than individuals without the mutation. Protein C activity levels correlated highly with APC-PCI complex levels (n=195; r_s=0.69; 95% CI 0.61-0.76). Figure 1 shows the scatterplot for the protein C activity levels and the APC-PCI complex levels with the exclusion of 2 subjects with APC-PCI complex concentrations of >1µg/L. The exclusion of these 2 individuals did not change the correlation coefficient (n=193; r_s=0.68; 95% CI 0.59-0.75). The correlation was almost similar in family members with the protein C 3363C insertion (n=55; r_s=0.50; 95% CI 0.26-0.68) and family members without the protein C 3363C insertion (N=140; r_s=0.44; 95% CI 0.29-0.57).

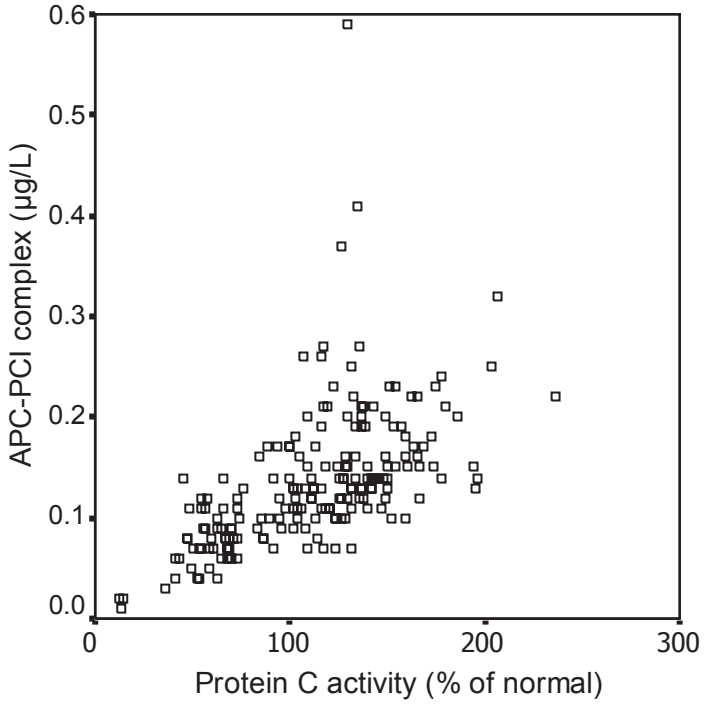
The levels of APC-PCI complex were not different between carriers and non-carriers of the prothrombin G20210A mutation (Table 1), those with or without a history of venous thrombosis, or between men and women (data not shown).

Table 1 *APC-PCI complex levels (µg/L) of the family members*

		APC-PCI complex	
	n	Mean (95% CI)	Range
Protein C 3363C insertion	55	0.08 (0.07-0.09)	0.01-0.17
+ PT G20210A	11	0.08 (0.07-0.10)	0.03-0.13
- PT G20210A	44	0.08 (0.07-0.09)	0.01-0.17
No protein C 3363C insertion	140	0.18 (0.15-0.21)	0.07-2.20
+ PT G20210A	13	0.19 (0.14-0.23)	0.10-0.37
- PT G20210A	127	0.18 (0.14-0.24)	0.07-2.20

Abbreviations: APC-PCI=activated protein C-protein C inhibitor, CI=confidence interval, PT=prothrombin

Figure 1 *Scatterplot of APC-PCI complex levels and protein C activity levels for 193 family members with or without the protein C 3363C mutation (excluding 2 individuals with APC-PCI complex levels above 1 µg/L)*



Discussion

The prothrombin G20210A variant is clearly associated with an increased risk for venous thromboembolic disease[18,23-25], as are the higher plasma levels of prothrombin associated with the A-allele.[8,18,26,27] Thrombin generation, as reflected by prothrombin fragment 1+2 plasma concentration, varies directly with prothrombin concentration.[11,12] This latter observation fits well with the increased risk of thrombosis associated with the mutation and raises questions with respect to the finding that the mutation does not confer risk in the presence of protein C deficiency in this French Canadian family.[11] However, the findings of the present study do not support our hypothesis of increased protein C activation resulting from higher prothrombin concentrations associated with the prothrombin G20210A polymorphism. It is possible that the plasma concentration of APC-PCI does not reflect APC production, but the positive correlation between plasma protein C levels and APC-PCI does not support this explanation. The only described situation in which APC-PCI does not reflect APC production is when PCI has been depleted, such as in seriously

ill septic or DIC patients.[28] Thus, our findings suggest that if the observed higher levels of thrombin generation and activity compensate for the impaired protein C pathway in this thrombophilic family it must be by an alternative mechanism.

Thrombin plays multiple roles in coagulation, fibrinolysis, platelet activation, cell growth, peripheral blood cell activation, anticoagulation, vascular endothelium and cell migration. Thus, the interaction of the prothrombin G20210A polymorphism with protein C deficiency in this thrombophilic kindred may not directly involve the protein C system. A recently described thrombin-mediated endothelial cell dependent mechanism for factor Va inactivation is a possible alternative mechanism.[29] However, as is the case with this multifunctional protein, thrombin has also been shown to inhibit the inactivation of Factor Va by activated protein C in purified systems.[30] Thus, we are left with an apparently paradoxical interaction of two well-established risk factors and an opportunity to learn more about the tonic thrombohemorrhagic balance first postulated by Åstrup in 1958.[31]

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Chapter II

Gene mapping in a protein C deficient family

Chapter II.1

Heritability of plasma concentrations of clotting factors and measures of a prethrombotic state in a protein C deficient family

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Summary

Earlier studies found strong support for a genetic basis for regulation of coagulation factor levels and measures of a prethrombotic state (D-dimer, prothrombin fragment 1.2). We wanted to estimate how much of the variation in the levels of coagulation factors and measures of a prethrombotic state, including measures of protein C activation and inactivation, could be attributed to heritability and household effect. Blood samples were collected from 330 members of a large kindred of French-Canadian origin with type I protein C deficiency. Heritability and common household effect were estimated for plasma concentrations of prothrombin, factor V, factor VIII, factor IX, fibrinogen, von Willebrand factor (vWF), antithrombin, protein C, protein S, protein Z, protein Z-dependent protease inhibitor (ZPI), fibrinopeptide A (FPA), protein C activation peptide (PCP), activated protein C-protein C inhibitor complex (APC-PCI), activated protein C-α1-antitrypsin complex (APC-α1AT), prothrombin fragment 1.2 (F1.2) and D-dimer, using the variance component method in SOLAR. The highest heritability was found for measures of thrombin activity (PCP and FPA). High estimates were also found for prothrombin, factor V, factor IX, protein C, protein Z, ZPI, APC-PCI and APC-α1AT. An important influence of shared household effect on phenotypic variation was found for vWF, antithrombin, protein S and F1.2. In conclusion, we found strong evidence for the heritability of single coagulation factors and measures of a prethrombotic state. Hemostatic markers with statistically significant heritability constitute potential targets for the identification of novel genes involved in the control of quantitative trait loci.

Introduction

Over a century ago, Virchow postulated that thrombosis was caused by alteration in the vessel wall, blood flow or the composition of the blood.[1] Several hereditary prothrombotic defects have been identified in the last four decades associated with a change in the composition of the blood. The first hereditary defects described were mutations in clot-preventing factors (antithrombin, protein C and protein S).[2-4] In 1994 and 1996, two highly prevalent mutations, the factor V Leiden mutation (FV G1691A) and the factor II G20210A mutation, were reported.[5,6] The latter two mutations are associated with resistance to inactivation of activated factor V and elevated concentrations of prothrombin, respectively. Elevated concentrations of other procoagulant factors, such as fibrinogen, factor VIII, factor IX and factor XI have been shown to increase the risk of venous thrombosis.[7-10] Several polymorphisms are known to influence plasma levels of fibrinogen.[11] For high levels of factor VIII, evidence for a genetic determination by factors other

than blood group and von Willebrand factor has been found.[12,13] However, no polymorphisms have been discovered yet in the factor VIII gene that can account for high levels of factor VIII.[14] For factor IX and factor XI it is still unclear whether genetic factors contribute or not, and to what extent. Recent studies have demonstrated a genetic basis for the regulation of plasma concentrations of coagulation factors and markers of a prethrombotic state (D-dimer and F1.2) by estimating heritability within healthy individuals or relatives of individuals with arterial or venous thrombosis.[15-18] Previously, we published preliminary data demonstrating strong evidence for the heritability of levels of markers of protein C activation and inactivation in a large family from French Canadian descent with type I protein C deficiency.[19]
The present paper describes the finalized analysis on the heritability of levels of coagulation factors and measures of a prethrombotic state with the addition of important information on household influence. Heritability and common household effect were estimated for plasma concentrations of prothrombin, factor V, factor VIII, factor IX, fibrinogen, von Willebrand factor (vWF), antithrombin, protein C, protein S, protein Z, protein Z-dependent protease inhibitor (ZPI), fibrinopeptide A (FPA), protein C activation peptide (PCP), activated protein C-protein C inhibitor complex (APC-PCI), activated protein C-α1-antitrypsin complex (APC-α1AT), prothrombin fragment 1.2 (F1.2) and D-dimer.

Materials and methods

Participants and inclusion criteria

Blood samples were collected from 330 members of a large kindred of French-Canadian origin with type I protein C deficiency, including spouses of family members with children. The ascertainment and evaluation of the family members was previously described.[20] All subjects completed questionnaires and were personally interviewed regarding their medical history in general, their risk factors for thrombosis (e.g., use of birth control pills, pregnancy, surgery, trauma, infection) and their thrombosis history. We classified a history of venous thrombosis as verified when subjects were hospitalized and treated for venous thrombosis with an objectively diagnosed deep venous thrombosis or pulmonary embolism. All participating subjects gave informed consent, or if individuals were under 18, a parent or legal guardian gave informed consent. We excluded from all analyses women pregnant at the time of the blood draw (n=5). We also excluded individuals using coumadin derivatives (n=15) for vitamin K dependent factors (including all measures of a prethrombotic state), as well as individuals with fibrinogen levels below 100 mg/dl (n=3) and factor V levels below 33 U/dl (n=1). This study was approved by the Human Experimentation Committees of the University of Vermont College of Medicine,

Burlington (VT, USA) and the Beth Israel Hospital, Boston (MA, USA).

Blood collection and processing

Peripheral blood was collected into siliconized glass Vacutainer tubes containing two different anticoagulants: 3.8% buffered citrate solution (Becton Dickinson, Franklin, NJ), and SCAT-1 (25 µM PPACK, 200 kIU/mL Aprotinin, 4.5 mM EDTA, Haematologic Technologies, Essex, VT). Platelet poor plasma from freshly drawn whole blood was produced within one hour by centrifugation at 3,000 x *g* for 10 minutes at room temperature and stored at –70ºC. Frozen plasma samples were thawed at 37ºC just before assay performance.

Assay methodology

All assays were performed in the investigators' laboratories either by a clot-based functional assay for protein C (coefficient of variation (CV) 5.5%)[20,21], by ELISA: factor V antigen (CV 5.8%)[22], factor VIII antigen (CV 7.8%), factor IX antigen (CV 10%), vWF (CV 3%)[23], protein Z (CV 6.5%)[24], ZPI (CV 7.2%)[24], APC-α1AT complex (CV 12.4%)[25] and APC-PCI complex (CV 11.7%)[25], or radio-immuno-assay: prothrombin (CV 8%)[26], antithrombin (CV 5%)[27,28], protein S (CV 9.8%)[29], F1.2 (CV 8%)[28], PCP (CV 14%)[28] and FPA(CV 8%)[28], the Clauss-method for fibrinogen (CV 1.7%)[30,31] using the ST4 instrument (Diagnostica Stago, Parsipanny, NJ, USA), or micro latex bead agglutination for D-dimer (CV 9.2%) (Biomerieux, Durham, NC, USA)[32,33].
Factor V antigen analysis was performed with an in house assay.[22] The kits for factor VIII antigen, factor IX antigen, protein C and vWF were provided by Diagnostica Stago (Parsippany, NJ, USA), and assays were performed following manufacturer's instructions. The assays for activated protein C-PCI complex and APC-α1AT complex were performed using commercially available assay at Affinity Biologicals Inc. (Hamilton, Ontario, Canada).
The number of individuals per assay varied, based on the availability of appropriate stored samples from 83 to 287.

Statistical analysis

To reduce skewness and kurtosis, we applied log transformation to the levels of factor V, vWF, antithrombin, FPA, PCP, APC-α1AT complex, APC-PCI complex and D-dimer, square root transformation to the levels of factor VIII antigen and F1.2, and reciprocal transformation to the levels of factor IX antigen. Heritability, the proportion of the phenotypic variance attributed to polygenes, and common household effect, the proportion of the variance attributed to environmental factors shared within a household, were estimated for each variable using the variance component method in SOLAR.[34] In addition we studied the effect on the heritability and household estimates of excluding

individuals with a definite venous thrombotic history, the protein C 3363C insertion and the prothrombin G20210A mutation. As only 3 family members carried the factor V Leiden mutation, we did not study the effect of excluding individuals with this mutation. The distribution of each (transformed) variable was assumed to be multivariate normal with a variance-covariance matrix following the formula: covariance (one person to another person)=h^2K + c^2H + e^2I, with K derived from the kinship matrix, H from the household matrix and I from the identity matrix. The additive genetic and household components of variance were estimated using maximum likelihood analysis. The adjustment for covariates was made as part of the heritability analysis. All analyses were adjusted for age and sex. We also adjusted for the use of oral contraceptives or hormone replacement therapy for prothrombin, factor V, factor VIII antigen, fibrinogen, vWF, protein S and C, antithrombin, and all measures of a prethrombotic state. In addition, levels of vWF were adjusted for ABO blood group and levels of factor VIII antigen were adjusted for ABO blood group and in some analyses for vWF.

Results

The mean age of the 322 family members at the blood draw was 31 years (range 1-90), 131 (41%) were men and the mean body mass index (kg/m^2) was 24.7 (range 12-52). The main characteristics of the 322 family members are outlined in Table 1. In total, 25% carried the 3363C insertion in the protein C gene, 13% carried the prothrombin G20210A polymorphism and 1% carried the factor V Leiden mutation, as confirmed by individual genotyping, and 9% had experienced a definite venous thrombotic event. The mean level of prothrombin was 128.4 U/dl in individuals with the G20210A mutation (range 90.7 to 165.0 U/dl).
Table 2 shows the mean level, standard deviation and range of all coagulation factors and measures of a prethrombotic state. The mean level and 95% CI ranges of levels of coagulation factors and measures of a prethrombotic state were largely in accordance with normal ranges found in our laboratory or by others[20,35,36] We found slightly high levels for D-dimer in 7 family members. For factor VIII antigen levels we found a broad range, but most individuals with lower levels of factor VIII had correspondingly low levels of vWF and tended to have blood type O.

Heritability and household estimates of single coagulation factors or markers

Table 3 shows heritability and household effect estimates for all coagulation factors and measures of a prethrombotic state. Among procoagulant factors, we found high heritability estimates for prothrombin (69.6%), factor V (71.4%) and factor IX antigen (50.3%) and lower heritability for fibrinogen

Table 1 *Main characteristics of all subjects*

Characteristics at blood draw* (n=322)	
Age (years;range)	31.3 (1-90)
Number of households	181
Mean number of individuals per household (range)	1.8 (1-6)
Spouses	21
Sex (M/F)	131/191
Body mass index (kg/m^2;range)	24.7 (12-52)
Smokers (age >13)	72/246 (29%)
Personal history of venous thrombosis (possible and definite events)	48/322 (15%)
Personal history of venous thrombosis (definite events)	28/322 (9%)
Anticoagulation treatment	15/322 (5%)
Hormone replacement therapy	10/181 (6%)
Oral contraceptive use	18/183 (10%)
Blood group O	80/290 (28%)
Protein C 3363C insertion	79/317 (25%)
Prothrombin G20210A mutation	38/293 (13%)#
Factor V Leiden mutation	3/302 (1%)

*Except for age, sex and body mass index, all characteristics are given as the number and percentage of individuals with the characteristic, including the number of individuals for whom information was available.
One individual was homozygous for the prothrombin G20210A mutation.

(29.7%), vWF (25.3%), and factor VIII antigen when adjusted for vWF (19.5%). Interestingly, a household effect was found for vWF (30.7%), but not for factor VIII antigen (unadjusted for vWF) despite the relationship between factor VIII and its carrier protein vWF. Significant heritability estimates for anticoagulant factors were found for protein C (40.6% for all tested individuals; 38.5% excluding those with the 3363C insertion, and 5.2% in those with the 3363C insertion), protein Z (66.7%) and ZPI (42.8%). The estimates of heritability were low for antithrombin (6.1%), and protein S levels (10.5%), while for both substantial household effects were found (respectively 33.5% and 36.9%). For measures of the activation and inactivation of protein C high heritability estimates were found for PCP (75.4%) and the APC-inhibitor complexes (58.6% for APC-α1AT and 58.4% for APC-PCI). FPA showed a high heritability (92.0%) likely reflecting the activity of thrombin. Heritability estimates, however, were low for prothrombin activation (F1.2; 22.1%) and D-dimer levels (6.6%), although for F1.2 a high household effect was found (44.1%).

Table 2 *Mean levels, standard deviation and range of all analytes*

Analytes (units)	n	Mean	SD	Range
Prothrombin (U/dl)	164	104.91	20.38	71.00-165.00
Factor V (U/dl)	259	117.75	37.13	54.76-299.75
Factor VIII antigen (U/dl)*	198	116.98	42.06	44.88-250.00
Factor IX antigen (U/dl)	222	93.08	30.69	52.87-250.00
Fibrinogen (mg/dl)	283	273.08	67.78	140.00-573.00
vWF (U/dl)	236	115.99	45.92	31.45-300.00
Antithrombin (% of normal)	176	98.23	16.52	64.10-161.60
Protein C (% of normal)	267	90.16	31.30	16.00-203.00
Protein C (3363C insertion only; % of normal)	59	50.68	15.11	16.00-109.00
Protein S (total; µg/ml)	163	15.35	2.48	8.00-24.10
Protein Z (% of normal)	274	104.50	36.02	21.00-237.00
ZPI (% of normal)	287	100.33	29.96	30.00-215.00
FPA (nM)	147	1.43	1.03	0.26-6.65
PCP (pmol/L)	83	1.33	0.74	0.25-4.17
APC-α1AT complex (nM)	164	0.28	0.49	0.01-4.30
APC-PCI complex (nM)	164	0.06	0.06	0.01-0.43
F1.2 (nmol/L)	147	2.28	1.56	0.40-10.20
D-dimer (ng/ml)	263	154.40	281.18	4.82-2551.48

Abbreviations: vWF=von Willebrand factor, ZPI=protein Z-dependent protease inhibitor, FPA=fibrinopeptide A, PCP=protein C activation peptide, APC-α1AT=activated protein C-α1-antitrypsin, APC-PCI=activated protein C-protein C inhibitor, F1.2=prothrombin fragment 1.2. *Only individuals for whom blood type was known.

No major differences (>30%) in heritability estimates or household estimates were seen after excluding family members with a definite history of venous thrombosis. However, we did find an increase or decrease of more than 30% in the heritability or household effects of several analytes after excluding individuals with the 3363C protein C gene insertion or the prothrombin G20210A mutation. Excluding individuals with the 3363C protein C mutation increased heritability and decreased the household effect for antithrombin to respectively 41% and 2.5%, and increased the household effect of prothrombin to 36%. Excluding individuals with the prothrombin G20210A mutation decreased heritability for prothrombin to 28% and increased heritability for APC-α1AT to 89%.

Table 3 *Proportion of phenotypic variance explained by covariates, heritability (h^2) and household effect (c^2)*

Analytes	N	Proportion of variance %				
		covariates	h^2	SE (h^2)	c^2	SE (c^2)
Prothrombin	164	11.7	69.6*	18.1	4.1	15.0
Factor V	259	14.8	71.4*	13.3	2.8	9.5
Factor VIII antigen (adjusted for vWF)	187	46.1	19.5*	15.9	6.6	10.9
Factor VIII antigen	198	26.9	30.9*	15.2	0.0	N/a#
Factor IX antigen	222	11.3	50.3*	19.0	3.3	12.0
Fibrinogen	283	24.3	29.7*	13.6	0.0	N/a#
vWF	236	19.9	25.3*	16.4	30.7*	11.1
Antithrombin	176	23.1	6.1	13.4	33.5*	13.9
Protein C	267	3.3	40.6*	15.8	4.4	10.3
Protein S (total)	163	23.7	10.5	31.1	36.9*	21.7
Protein Z	274	0.0	66.7*	12.6	6.6	8.8
ZPI	287	7.4	42.8*	15.8	1.3	9.6
FPA	147	0.0	92.0*	15.9	0.0	N/a#
PCP	83	5.6	75.4*	29.6	0.0	N/a#
APC-α1AT complex	164	1.8	58.6*	22.3	4.8	14.2
APC-PCI complex	164	0.0	58.4*	18.0	0.0	N/a #
F1.2	147	29.6	22.1	20.3	44.1*	13.3
D-dimer	263	13.5	6.6	12.9	5.2	10.3

Abbreviations: SE=standard error, vWF=von Willebrand factor, ZPI=protein Z-dependent protease inhibitor, FPA=fibrinopeptide A, PCP=protein C activation peptide, APC-α1AT=activated protein C-α1-antitrypsin, APC-PCI=activated protein C-protein C inhibitor, F1.2=prothrombin fragment 1.2. *Significant at $p<0.05$. #No standard errors can be approximated for estimates of 0% or 100% in likelihood analysis.

Discussion

This study was performed to establish evidence for a genetic basis for plasma concentrations of hemostatic markers known to confer risk for thrombosis. The highest heritability estimates were found for the measures of thrombin activity (PCP and FPA). These constitute the best potential targets for the identification of novel genes involved in the control of quantitative trait loci. High heritability was also found for prothrombin, factor V, factor IX antigen, protein C, protein Z, ZPI, APC-PCI and APC-α1AT. Fibrinogen, vWF, antithrombin, thrombin generation (F1.2) and endogenous fibrinolysis (D-dimer) showed low heritability. Heritability was also low for protein S; however, protein S differs from the other vitamin-K dependent factors in that

it is not a protease and is for 60% bound to C4b-bp in plasma. The heritability estimate for factor VIII antigen was low after adjustment for vWF levels, probably due to the dependance of factor VIII levels on the stabilization of factor VIII in plasma mediated by factor VIII binding to vWF. For protein C we found a heritability of 40.6% for all tested individuals, a heritability of 38.5% after excluding those with the 3363C insertion, and a heritability of 5.2% in those with the protein C 3363C insertion. The latter suggests that due to the lower sample size and having 1 active allele instead of 2, by chance, other genes affecting protein C may not be present or may not affect individuals with lower levels of protein C.

An important influence of the shared household effect on phenotypic variation was found for vWF, antithrombin, protein S levels and F1.2. A household specific acute or chronic activation of the coagulation pathway by tobacco use, diet, physical activity or other shared environmental characteristics could explain the high household effects not only for F1.2 and vWF, but also for the anticoagulation factors protein S and antithrombin, as household specific differences in activation of the clotting system may give rise to compensatory differences in these inhibitors.

Several other studies have estimated heritability with regard to the coagulation system but focused primarily on procoagulant and anticoagulant factors.[15-18] We found high heritability for markers of thrombin activation (FPA and PCP) and the activated protein C-inhibitor complexes. In contrast F1.2, a marker of thrombin generation, did not demonstrate significant heritability but did show a significant household effect. Adjustment for F1.2 in the present study did not change the heritability of FPA, PCP and the activated protein C-inhibitor complexes. Thus, thrombin generation, a critical step in hemostasis, seems to be influenced largely by environmental factors. However, its activity as measured by FPA, PCP and complexes of activated protein C with its inhibitors appears to be tightly controlled through genetic mechanisms which may implicate proteins like thrombomodulin, the endothelial cell protein C receptor and fibrinogen. Table 4 gives a comparison of the heritability estimates of the most recently published studies and the present study.[15-18] The variance component method used to estimate heritability and household effect assesses the relative role of genetic and environmental causes of variation of a quantitative trait in a particular setting and population. Because genes and environment are different in various populations, heritability estimates cannot be readily compared quantitatively across populations. The populations shown in Table 4 are different not only by geography but also by selection of the sample from the population: De Souto *et al*.[15] estimated heritability in family members of probands from multiple families with hereditary thrombophilia in Catalonia, Spain, whereas the studies by Ariëns *et al*. and De Lange *et al*. [16,17] comprised healthy twins from the United Kingdom. We studied a single

kindred, which decreases both the genetic variance, due to familial genetic similarity, and the random environmental variance, due to greater similarity of lifestyles among different households within a family. Although the total variance is generally smaller in a single kindred compared to more varied populations, the increased genetic homogeneity facilitates the likelihood of detecting the gene(s) underlying the observed heritability. A potential source of error across populations could be attributed to the methods used to measure analytes with regard to the variation in pre-analytical and analytical errors, which could decrease heritability.[37] We did not account for non-additive sources of genetic variance like dominance and epistasis, which could also decrease heritability.

Table 4 *Heritability estimates (h^2,%) found in the present study and other studies*

Analyte	h^2, %			
	USA	UK-1[16,17]	UK-2[18]	Spain[15]
Prothrombin	70	57	Nd	49
Factor V	71	Nd	Nd	44
Factor VIII antigen	20*	61#	Nd	40#
Factor IX antigen	50	Nd	Nd	39
Fibrinogen	30	44	35	34
vWF	25	75	Nd	32
Antithrombin	6	Nd	Nd	49
Protein C	41	Nd	Nd	50
Protein S	11	Nd	Nd	46
Protein Z	67	Nd	Nd	Nd
ZPI	43	Nd	Nd	Nd
FPA	96	Nd	Nd	Nd
PCP	75	Nd	Nd	Nd
APC-α1AT complex	59	Nd	Nd	Nd
APC-PCI complex	58	Nd	Nd	Nd
F1.2	22	45	Nd	Nd
D-dimer	7	65	Nd	11

Abbreviations: vWF=von Willebrand factor, ZPI=protein Z-dependent protease inhibitor, FPA=fibrinopeptide A, PCP=protein C activation peptide, APC-α1AT=activated protein C-α1-antitrypsin, APC-PCI=activated protein C-protein C inhibitor, F1.2=prothrombin fragment 1.2, Nd= not determined. *Adjusted for ABO blood group and vWF. #Not adjusted for ABO blood group and vWF.
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The heritability estimates in this paper are only slightly different from the estimates we published earlier,[19] and differ mainly due to the estimation of household effects. A shared household effect apparently seems to explain a part of the heritability previously published for levels of vWF, antithrombin and protein S,[19] showing the importance of estimating household effects as part of a variance component analysis. Differences between studies in Table 4 could thus also be attributable to the magnitude of the household effect.
Exclusion of individuals with the 3363C insertion or the prothrombin G20210A mutation influenced few heritability and household estimates of coagulation factors or activation markers. An increase in the household effect after removal of carriers of a certain gene or trait could be the result of a decrease in the total variance, so that the household component accounts for more of the total variance. We have no explanation for the increase in heritability estimates after removal of carriers of a gene or trait other than that the inheritance of other genes fitted the polygenic model better once the gene-carriers were removed. However, these changes can be used in defining the most informative subsets in subsequent quantitative trait loci linkage analysis.
In conclusion, we found strong evidence for the heritability of single coagulation factors and measures of a prethrombotic state. Hemostatic markers with statistically significant heritability constitute potential targets for the identification of novel genes involved in the control of quantitative trait loci.[38,39]

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Chapter II.2

Evidence for several loci influencing protein C activity

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Summary

Previously we found a genetic basis for the variation in the plasma concentrations of several coagulation factors in a large pedigree with protein C deficiency. In addition, we estimated high heritabilities for markers of a prethrombotic state, including the plasma concentration of complexes of activated protein C (APC) with its main inhibitors α1-antitrypsin (α1AT) and protein C inhibitor (PCI). Performing variance component linkage analysis on the same pedigree, we found suggestive evidence for linkage (defined as a lod score between 1.9 and 3.3) on chromosome 19 at 34 cM (lod score 3.29) for plasma concentrations of APC-α1AT complex, and on chromosome 11 at 146 cM (lod score 2.55) for plasma concentrations of APC-PCI complex. The identified linkage peaks did not seem the result of variation in the plasma concentrations of single coagulation factors related to the complex, i.e. PCI, α1AT and protein C. The areas with the lod scores suggestive of linkage were identified by relatively few markers and did not reveal obvious candidate genes. Fine-mapping in these regions with additional markers will both validate and narrow the regions of interest on chromosomes 11 and 19.

Introduction

Protein C is a vitamin K dependent zymogen, which after activation by the thrombin-thrombomodulin complex on the surface of endothelial cells down-regulates the hemostatic system through the proteolytic inactivation of factors Va and VIIIa.[1,2] Activated protein C is inhibited primarily by the serine proteinase inhibitors α1-antitrypsin and protein C inhibitor.[3] Hereditary defects in the protein C gene have been associated with an increased risk of venous thrombosis[4-6]: a complex disease with an annual incidence of about 1 per 1000 persons, which manifests itself mainly in the deep veins of the leg or the lungs, and which can be caused by several genetic and environmental risk factors.[7,8] In 1989 we published the first data on a French Canadian kindred with an increased risk of venous thrombosis due to an insertion mutation in the protein C gene (3363_3364insC).[9] Recently, we showed high heritability in this French Canadian kindred for plasma concentrations of the procoagulant factors, factor V, factor IX, and prothrombin, and the coagulation inhibitors protein C, protein Z and protein Z-dependent protease inhibitor.[10,11] In addition, we found evidence for a genetic basis for measures of thrombin activity (fibrinopeptide A and protein C activation peptide) and plasma concentrations of protein C inhibitor complexes (activated protein C-protein C inhibitor complex (APC-PCI) and activated protein C-α1-antitrypsin (APC-α1AT) complex).[10,11] Our previous report demonstrated that over half of the variance in the APC-α1AT complex and APC-PCI complex plasma concentrations

could be attributed to genetic influences.[10,11] As this thrombophilic kindred has an altered protein C system, we focused on assessing the regulation of this system through evaluating the genetic components responsible for the greater than half of the variance in these two major inhibitors in complex with activated protein C. The GAIT study, a family based study focussing on the Genetic Analysis of Idiopathic Thrombosis, localized earlier several quantitative trait loci (QTLs) of traits relevant to thrombosis like prothrombin, factor VIII and activated protein C resistance.[12,13] Recently, they found evidence for a locus influencing normal variation in protein C levels on chromosome 16.[14] The present report describes the results of a variance component linkage analyses performed on plasma concentrations of APC-α1AT and APC-PCI complexes.

Methods

Participants and inclusion criteria

Plasma concentrations of APC-α1AT and APC-PCI complexes were measured in members of a large kindred of French-Canadian origin with type I protein C deficiency, including spouses of family members with children. The ascertainment and evaluation of all family members was previously described.[9] All subjects completed questionnaires and were personally interviewed regarding their general medical history, risk factors for thrombosis (e.g., use of birth control pills, pregnancy, surgery, trauma, infection) and their thrombosis history. All participating subjects or their legal guardians gave informed consent. This study was approved by the Human Experimentation Committee of the University of Vermont College of Medicine, Burlington (VT).

Blood collection, processing and assay methodology

Peripheral blood was collected into either of two siliconized glass Vacutainer tubes containing two different anticoagulants: 3.8% buffered citrate solution (Becton Dickinson, Franklin, NJ), or SCAT-1 (25 μM PPACK, 200 kIU/mL Aprotinin, 4.5 mM EDTA, Haematologic Technologies, Essex, VT). Platelet poor plasma from freshly drawn whole blood was produced within one hour by centrifugation at 3,000 x *g* for 10 minutes at room temperature and stored at –70ºC. Frozen plasma samples were thawed at 37ºC just before assay performance.

Plasma concentrations of APC-α1AT complex and APC-PCI complex were measured using a commercially available assay at Affinity Biologicals Inc. (Hamilton, Ontario, Canada). The inter-assay coefficients of variation (CVs) were 12%. The presence of the 3363C insertion in the protein C gene was determined by amplification of genomic DNA using a mutagenic oligonucleotide primer that in concert with the inserted C mutation creates a

*Bg1*I cleavage site. The product was digested with *Bg1*I and analyzed on a 2% agarose gel.[15] The G20210A allele was detected by amplification of genomic DNA with a mutagenic primer resulting in a *Hind*III cleavage site when the A-allele was present.[16] To test whether the identified linkage peaks might be the result of variation in the plasma concentrations of single coagulation factors related to the complex or to thrombin generation, we also performed variance component linkage analyses for plasma concentrations of protein C, α1AT, PCI and prothrombin fragment 1.2 (F1.2). We measured protein C activity levels with a clot-based functional protein C activity assay using the Staclot Protein C kit provided by Diagnostica Stago (Parsippany, NJ, USA). The inter-assay CV of this assay was 6%. Alpha1-antitrypsin plasma concentrations were measured performing rate nephelometry using a Beckman-Coulter Image with an inter-assay CV of 4%. Both the protein C activity and α1-antitrypsin assays were performed following manufacturer's instructions. PCI (PAI-3) plasma concentrations were measured performing an ELISA using a kit from Enzyme Research Laboratories (South Bend, IN, USA) with an inter-assay CV of 4%. Prothrombin activation fragment 1.2 (F1.2) concentrations were measured by a radio-immuno-assay with an inter-assay CV of 8%.[17]

Data analysis

Genotyping was performed with 375 autosomal markers by the NHLBI Mammalian Genotyping Service at the Marshfield Medical Research Foundation [18] using Screening Set version 10. Marker spacing averaged 9.4 cM (range 0-18 cM) with an average marker heterozygosity of 75% (range 42-89%). As described previously[19], genotype data were checked for pedigree and genotyping errors using Eclipse2[20] and Simwalk2[21,22], and the marker allele frequencies were estimated in PAP.[23] The probability of Identity By Descent (IBD) was estimated using the multipoint IBD method in Simwalk2[21] which uses the proportion of alleles shared identical by descent at marker loci to estimate IBD sharing at arbitrary points along the chromosome for each relative pair. Input files were produced using MEGA2.[24]

Subsequently, using SOLAR (Sequential Oligogenic Linkage Analysis Routines)[25], we performed variance component linkage analysis to test whether a proportion of the genetic variance in the plasma concentrations of APC-α1AT complex or APC-PCI complex could be attributed to specific genomic locations. The plasma concentrations of APC-α1AT and APC-PCI complexes were log-transformed to reduce skewness (from 5.4 to 0.0 for APC-α1AT; from 3.0 to 0.2 for APC-PCI) and kurtosis (from 33.9 to 0.4 for APC-α1AT; from 11.6 to 0.4 for APC-PCI). Of the levels of protein C we took the square root to reduce skewness from 0.4 to –0.1 (kurtosis went from 0.3 to -0.2), levels of PCI, α1AT and F1.2 were log-transformed to reduce skewness from,

respectively, 0.6 to –0.1, 0.6 to 0.0 and from 2.1 to 0.2. Kurtosis decreased from 1.0 to –0.1 for PCI, from 0.8 to 0.3 for α1AT (after removing 2 outliers) and from 5.9 to -0.1 for F1.2. All (transformed) levels were assumed to distribute as a multivariate normal density with correlation=$h^2K + c^2H + q^2B + e^2I$, where matrix K contains the kinship coefficients, H contains 1 for pairs from the same household and 0 otherwise, B contains the IBD probabilities, and I represents the identity matrix. The parameters for heritability (h^2), household effect (c^2), and heritability contributed to a specific genomic location (q^2) as well as the effects of the covariates were estimated simultaneously using maximum likelihood analysis. Lod scores were computed as the $\log_{10}$ likelihood for q^2 estimated to $q^2=0$. A lod score of 3.3 was used as cut-off point for statistical evidence for significant linkage as suggested by Lander and Kruglyak, and a lod score of 1.9 but below 3.3 for suggestive linkage evidence.[26] By including covariates in the linkage model, which might absorb some of the variation, we tried to increase the proportion of variation due to a QTL. These covariates were characteristics that have a documented effect on the activation state of the hemostatic system: age, sex, use of oral contraceptives or hormone replacement therapy, and presence of the protein C mutation.[17,27-29] Individuals on coumarin derivatives (n=15), women pregnant at the time of the blood draw (n=6) or women for whom we had no information on the use of female hormones (n=6) were not included in the analysis. Among the subjects on coumarin derivatives the mean APC-α1AT complex level was 0.09 nM (range 0.02-0.27; n=9), and the mean APC-PCI complex level was 0.02 nM (range 0.01-0.05; n=10).

Results

Of all genotyped family members who fulfilled the above criteria, 135 subjects were tested for plasma concentrations of APC-α1AT complex and 129 for plasma concentrations of APC-PCI complex (125 subjects were tested for both complexes). The main characteristics of the participants are depicted in Table 1. A preponderance of the participants was female. Table 2 shows the mean plasma concentrations of the plasma concentrations of APC-α1AT complex, APC-PCI complex, protein C activity levels, α1-antitrypsin, PCI and F1.2. The variance component linkage analysis based on the Marshfield map for the 22 autosomes revealed lod scores above 1 for chromosomes 3, 10, 11, 13, 18 and 19 for plasma concentrations of APC-α1AT (Table 3). The highest lod score was found for a region on chromosome 19 (34 cM Marshfield Map, nearest marker D19S586), showing evidence for linkage with a lod score of 2.77. In addition, a lod score of 2.01 was found on chromosome 13 (54 cM Marshfield Map, nearest marker D13S800); however, this lod score dropped to 1.29 after accounting for the region on chromosome 19 by computing a

Table 1 *Main characteristics of all participants at the time of the blood draw*

Characteristics	Subjects	
	APC-α1AT complex measured (n=135)	APC-PCI complex measured (n=129)
Mean age at blood draw (years (range))	28 (1-75)	29 (1-75)
Sex (Males/Females)	60/75	56/73
Number of households (n)	95	92
Individuals per household (mean (range))	1.4 (1-4)	1.4 (1-4)
Spouses (n)	0	0
Protein C 3363C insertion carriers (n (%))	28 (21)	25 (19)
Prothrombin G20210A mutation carriers (n (%))	28 (21)*	28 (22)*
Factor V Leiden mutation carriers (n (%))	0 (0)	0 (0)
Venous thrombosis history** (n (%))	20 (15)	19 (15)
Definite venous thrombosis history (n (%))	8 (6)	8 (6)
OCC users (age 10-40) (n/total n (%))	11/55 (20)	11/54 (20)
HRT users (age>40) (n/total n (%))	2/11 (18)	0/12 (0)

*One was homozygous for the prothrombin G20210A mutation. **Both possible and definite events.

Table 2 *Mean plasma concentrations, standard deviations and ranges of all analytes*

Analyte (units)	N	Mean	SD	Range
APC-α1-antitrypsin complex (nM)	135	0.30	0.53	0.01-4.30
APC-protein C inhibitor complex (nM)	129	0.07	0.06	0.01-0.43
Protein C activity (% of normal)	229	92.9	30.8	25.0-203.00
α1-antitrypsin (mg/dl)	124	132.7	24.8	79.0-214.0
Protein C inhibitor (% of normal)	150	151.4	36.8	74-301
F1.2 (nmol/L)	119	2.4	1.7	0.4-10.2

Abbreviations: SD=standard deviation, F1.2=prothrombin fragment 1.2

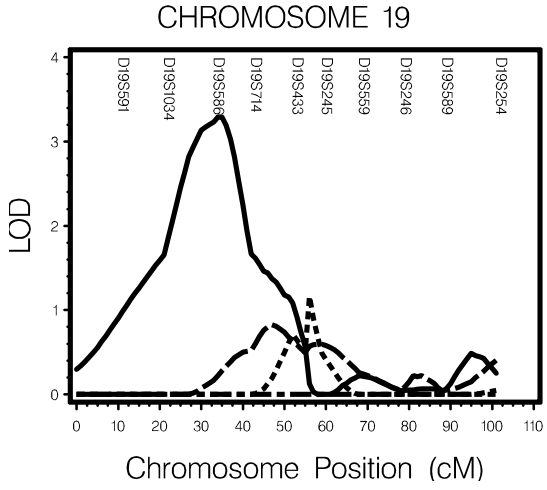
lod score relative to the chromosome 19 maximum. Including the covariates age, sex and female hormones in the model resulted in a lod score of 3.29 at 34 cM at chromosome 19 with a heritability estimated at 77% (standard error 23%), no apparent household effect (0%), and with 2% of the total variance explained by the covariates. Adding protein C mutation status as a covariate did not drastically lower the lod score (lod score: 3.0). The removal of family members with verified venous thrombosis (n=8) decreased the lod-score to 2.60 at chromosome 19 (at 35 cM; accounting for the effect of age, sex and use of female hormones). Figure 1 shows the peak on chromosome

Table 3 *Lod scores above 1 for APC-a1-antitrypsin complex.*

Chromosome	Closest marker	Location	Lod Score
3	D3S3630	11	1.07
10	D10S1248	168	1.13
11	ATA34E08	29	1.51
13	D13S800	54	2.01
18	D18S1371	114	1.15
19	D19S586	33	2.77

19 on all 135 subjects in detail. The 95% confidence interval around the peak, approximated by a one-unit lod score drop on both sides of the peak, ranged from 25 cM to 38 cM.

Figure 1 *Lod scores on chromosome 19 for APC-a1-antitrypsin complex (solid), protein C (dashed; peak at 47 cM), a1-antitrypsin (dashed; no peak) and F1.2 (dashed; peak at 56 cM) adjusted for age, sex, and female hormones*



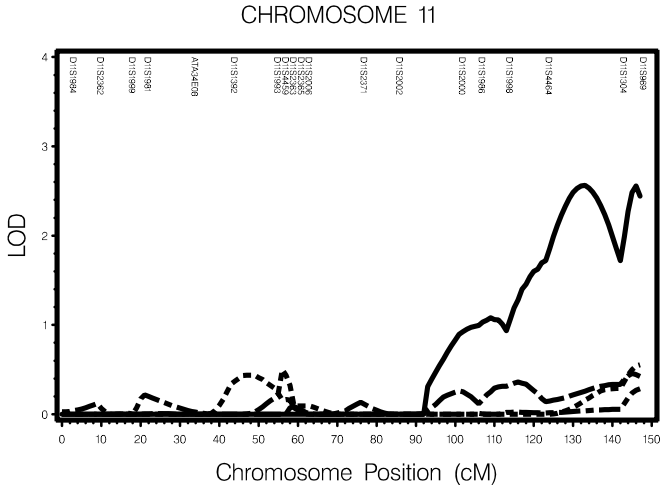
The variance component linkage analysis performed for plasma concentrations of APC-PCI complex estimated lod scores above 1 on chromosomes 5,11,17 and 18 (Table 4), all of which were below a lod score of 1.9, the cut-off point for suggestive linkage evidence. Including age as a covariate increased the lod score on chromosome 11 to 2.08 with a heritability estimated at 53% (standard error 20%), no apparent household effect (0%) and with 2.8% of the variance explained by age. The removal of family members with venous thrombosis (n=8) increased the lod-score to 2.55 (with age included in the

Table 4 *Lod scores above 1 for APC-PCI complex*

Chromosome	Closest marker	Location	Lod Score
5	D5S2500	73	1.73
11	D11S969	145	1.78
17	D17S2196	39	1.27
18	D18S877	51	1.73

model) at chromosome 11 (146 cM). Figure 2 shows the peak for chromosome 11 in the 121 subjects without venous thrombosis. The 95% confidence interval around the peak ranged for the region on chromosome 11 from 119 to 147 cM.

Figure 2 *Lod scores on chromosome 11 for APC-PCI (solid), protein C (dashed; no peak), PCI (dashed; peak at 56 cM) and F1.2 (dashed; peak at 48 cM) adjusted for age in subjects without a history of venous thrombosis.*



Plasma concentrations of protein C, α1AT and thrombin activation might affect the plasma concentrations of APC-α1AT and APC-PCI. Levels of protein C and prothrombin fragment 1.2 had been measured already in some of the genotyped family members. In addition, we recently measured α1AT and PCI plasma concentrations. No evidence for linkage was observed on chromosomes 11 and 19 for protein C and F1.2 plasma concentrations, and on chromosome 11 for PCI and chromosome 19 for α1AT plasma concentrations, using the same exclusion criteria and adding the same covariates for both complexes as in the above analysis (Figures 1 and 2).

Discussion

Variance linkage component analysis identified a quantitative trait locus on chromosome 19p13 influencing the plasma concentration of APC-α1AT complex (lod score 3.29; 34 cM), and a quantitative trait locus on chromosome 11q25 influencing the plasma concentration of APC-PCI complex (lod score 2.55; 146 cM). Thus, although both of these serine protease inhibitors (serpins) are closely related and are coded in the same cluster on 14q32.1, we found different chromosomal regions influencing levels of APC in complex with one of these inhibitors. Although the two inhibitors are structurally related, they differ functionally as demonstrated by the finding that PCI activity can be regulated by heparin in contrast to α1AT activity.[30] In addition, α1AT contains a single reactive site centered at a Met^{358}-Ser^{359} sequence 36 amino-acid residues from the C-terminus, whereas for PCI the reactive site peptide bond is located at Arg^{354}-Ser^{355}. We previously reported evidence for linkage of thrombosis in region 11q23 (106 cM; p=0.043), which is relatively distant from the region found for APC-PCI complex (146 cM), and found no evidence for linkage with thrombosis on chromosome 19 (p-value on chromosome 19 in that area ranged from 0.80 to 0.95).[19] Both complexes are likely to be intermediate phenotypes for venous thrombosis according to a study by España et al[31], who found high levels of both complexes in patients with deep venous thrombosis. However, Watanabe et al[32] only found increased levels of APC-PCI complex and not APC-α1AT complex in patients with deep venous thrombosis and pulmonary embolism.

Genes regulating plasma concentrations of protein C or the activation of protein C could have influenced the plasma concentrations of APC-α1AT complex and APC-PCI complex. However, it seems unlikely that plasma concentrations of protein C influenced the plasma concentrations of APC-α1AT and APC-PCI complex for the following reasons: the gene for protein C lies on chromosome 2, accounting in the analyses for the effect of the protein C 3363C mutation did not dramatically decrease the lod score and linkage was not observed for protein C plasma concentration on either chromosome 11 or 19. The plasma concentrations of the inhibitors might determine some of the variance in the concentrations of the complexes. Local regulatory events involving control of transcription appear to be excluded by the absence of linkage on chromosome 14 where the genes for α1AT and PCI are located, and no linkage evidence was found for either PCI or α1AT on, respectively, chromosome 11 and chromosome 19. Plasma concentrations of free α1AT or PCI could also have been influenced through complex formation with target proteases other than APC, or by inactivation of free α1AT or PCI by metalloproteases. No obvious candidate genes were found in the regions on chromosomes 11 and 19 that would influence clearance or post-translational

processing of protein C, α1AT or PCI.
As specific candidate genes were not identified in the regions of linkage, it appears that yet to be determined genes in these regions explain over half the variance in these protease inhibitor complexes. The next stage of this study will be to fine-map the locations identified on chromosomes 11 and 19 to validate the quantitative trait loci and narrow the regions containing potential candidate genes.

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Summary

Rationale and aims of this thesis

Venous thrombosis is a common disease, which manifests mostly in the deep veins of the leg, with a reported incidence of 1-2 per 1000 individuals per year.[1,2] Several genetic risk factors have been identified for venous thrombosis: mutations in the natural coagulation inhibitors protein C, protein S and antithrombin, and in the procoagulant factors prothrombin and factor V.[3-7] It is, however, difficult to predict the risk of venous thrombosis for carriers of these defects, as venous thrombosis manifests itself as a multicausal disease, in which multiple genetic and environmental factors play a role and can interact in the onset of disease.[8-10] In families with a clear tendency to develop venous thrombosis (thrombophilia) the presence of multiple genetic risk factors explains the higher risk in individuals from these families compared with individuals with the same defect without a family history of venous thrombosis.[11,12] Large studies on the risk of venous thrombosis associated with familial thrombophilia are scarce, mainly because families with defects in natural anticoagulant inhibitors are rare. This has precluded the formulation of rational guidelines on thrombophilia testing and the duration of anti-coagulation treatment in these families. We wanted to address this paucity of evidence by initiating a prospective cohort study including individuals with inherited thrombophilia from different European thrombosis centers with expertise on thrombophilia research: the EPCOT study. The first part of this thesis (Chapter I) mainly describes the results of this study regarding the risk of a first or recurrent venous disease or a first fetal loss in carriers of known familial defects.

According to the literature, only in 13% of the thrombophilia families at least two genetic factors can be found, whereas in 60% of the families one genetic risk factor and in 27% of the families no risk factor can be found[13], despite the belief that families with a high tendency to venous thrombosis carry multiple defects.[11,12] Thus, the currently known genetic defects can explain only partly the increased risk of venous thrombosis in thrombophilic families. This was underlined by the evidence found in a large kindred with protein C deficiency for the presence of an unknown second major gene defect, which increased the risk of venous thrombosis in interaction with the protein C 3363C mutation.[14] Chapter II focuses on finding new genetic risk factors for venous thrombosis in the protein C deficient kindred by searching for genes regulating intermediate hemostatic phenotypes like plasma concentrations of factor VIII.

Results and general discussion

Inherited thrombophilia and the risk of venous thrombosis and fetal loss: the EPCOT study

A total of 2838 participants were included in the EPCOT study: 1626 with familial thrombophilia, and 1212 friends or partners as controls (representing the general population). Chapter I.1 describes the lifetime risk of venous thrombosis in all subjects (thrombophilia and control subjects) before they entered the EPCOT study. In total, 600 probands, the first of a family identified with a prothrombotic defect, and 846 relatives with a genetic defect were included in the analysis. Of these thrombophilic subjects and the 1212 controls, 532 probands (89%), 139 relatives (16%) and 15 controls (1%) had experienced a venous event in the deep veins of the legs or the lungs before study entry. Recurrent events already had occurred in 288 probands (54%), 58 relatives (42%) and 2 controls (13%). The incidence of venous events experienced before study entry in relatives was 4.4 (95% CI 3.7-5.2) per 1000 person years and in controls 0.3 (95% CI 0.2-0.5) per 1000 person years with a relative risk of 16.4 (95% CI 9.6-28.0), adjusted for sex and center effects (Table 1). Considerable differences in the lifetime risk of venous thrombosis were found among individuals with different thrombophilic defects. We found the highest incidence of events in the relatives with combined defects (8.4 per 1000 person years), as has been described by other authors.[11,15-18] For single defects we found the highest risk in subjects with protein S deficiency (7.1 per 1000 person years), and the lowest risk was associated with factor V Leiden (1.5 per 1000 person years), although several others have shown that the greatest venous thrombotic risk is associated with antithrombin deficiency.[19-22] Obviously, we might have underestimated the risk of venous thrombosis as we only counted follow-up years for subjects who had entered in the cohort, and therefore individuals, who had died of a fatal venous event before the start of the study were not included. However, in previous studies we have shown that the mortality of antithrombin deficiency[23], protein C deficiency[24] and factor V leiden[25] does not exceed the population risk.

Chapter I.2 shows the prospective data on the risk of a first venous event in subjects with thrombophilia, which was analyzed in 575 thrombosis-free relatives and 1118 controls receiving no long-term (i.e. for more than one year) anticoagulation treatment during prospective follow-up. In total, 26 thrombophilic subjects (4.5%) and 7 controls (0.6%) experienced a first venous thrombotic event in the deep veins or in the lungs during prospective follow-up. In 15 thrombophilic individuals (58%) and 3 controls (43%) these events occurred spontaneously, i.e., in the absence of established risk factors such as surgery, pregnancy or immobilization. The incidence of a first deep venous thrombosis or pulmonary embolism was 0.8% per year in relatives

compared with 0.1% per year in controls with a relative risk of 9.0 (95% CI 3.8-21.1), adjusted for sex, age and center effects (Table 1). Again, the incidences differed considerably for different thrombophilia defects: it was highest for antithrombin deficiency (1.7%/year) and combined defects (1.6%/year), and again lowest for factor V Leiden (0.1%/year). Reports from other studies include higher, similar and also lower risks for the various defects, which might be due to differences in study design.[17,19-22,26-32] Overall, we found a higher incidence of a first event in the prospective analysis than in the retrospective analysis (Table 1). Since the follow-up time for the retrospective study was from birth until inclusion in the study, the incidence rates will have been diluted as the risk of venous thrombosis until the age of 15 is very low. In contrast, the relative risk of a first venous event associated with thrombophilia was higher in the retrospective analysis than in the prospective analysis (Table 1). On the one hand, this could be due to mainly selecting symptomatic relatives at inclusion, which increases the incidence rates of the thrombophilia subjects in the retrospective analysis, and on the other hand the incidence rates in the prospective analysis could have been lower due to increased care for those subjects. Since no inception cohort was formed for the retrospective analysis, we consider the prospective design as more reliable.

Chapter I.3 describes the recurrence rate of venous thrombosis during prospective follow-up in all patients, both probands and relatives, with a thrombophilic defect and only one deep venous thrombosis or pulmonary embolism before study entry. Forty-four of the 180 (24%) patients who did not receive long-term anticoagulation and 7 of the 124 (6%) patients who were on long-term anticoagulation experienced a recurrent deep venous thrombosis or pulmonary embolism. The recurrence rate in the absence of long-term anticoagulation was 5.0% per year (95% CI 3.6-6.7), and thus much higher than the incidence of a first event (Table 1). However, the overall recurrence rate was not much different from that found for consecutive patients with a first thrombotic event with reported incidences ranging from 13-28% over 5 years of follow-up.[33-36] As in Chapter I.2, the lowest rate was found in thrombophilia subjects with factor V Leiden, and the highest rate in subjects with antithrombin deficiency. When we restricted the analysis to men, who had a 3-fold higher risk of venous thrombosis than women, much higher recurrence rates (around 11% per year) were found in men with deficiencies of protein C, protein S, and those with multiple defects than in women (2-3% per year). For men and women with antithrombin deficiency the risk was similar (respectively 12% and 10% per year). The recurrence rate was 80% lower for patients on long-term anticoagulation (1.1% per year; 95% CI 0.4-2.2) and was almost similar to the incidence of first events in thrombophilic participants (Table 1), but the use of long-term anticoagulation treatment also

resulted in a risk of major hemorrhage of 0.8% per year.
From the results on the risk of venous events (Chapters I.1-3) we could not infer a clear benefit for long-term anticoagulation treatment in preventing a first event, as the risk of a first venous thrombosis (Table 1) was in general not substantially higher than the risk of major hemorrhages associated with anticoagulant therapy (requiring hospitalization or surgery, or leading to death), which is reported to lie between 1 and 3%.[37,38] In addition, the recurrence rate in thrombophilia patients was not higher than in consecutive patients, for whom long-term anticoagulation treatment is not recommended in clinical practice after a first event.[39-42] However, our study is an observational study, and only randomized trials can demonstrate whether the benefit from long-term treatment is indeed not sufficient. The EPCOT study can only give rough guidelines based on the absolute risks found, especially as the treatment policies differ per center, but the results can assist physicians in decisions on testing on thrombophilic abnormalities and choices about long-term anticoagulation after a first thrombosis. It is important to keep in mind that the results from the EPCOT study can only be generalized to individuals from thrombophilic families, but not to unselected individuals with the same defect, as we have previously shown that these individuals have a lower risk of thrombosis.[11,12]
A secondary aim of the EPCOT study was to study the risk of fetal loss in women with thrombophilia. Thirty-nine women with thrombophilia and 51 control women in the EPCOT study had not experienced a fetal loss (i.e. any loss during pregnancy) before study entry and did not use any anticoagulants during the first pregnancy in the prospective follow-up. Of these women, the first pregnancy during prospective follow-up ended in fetal loss in 7 of the 39 women with thrombophilia and 7 of the 51 control women (relative risk 1.4; 95% CI 0.4-4.7) (Chapter I.4). Per type of defect the relative risk varied only minimally from 1.4 for factor V Leiden to 1.6 for antithrombin deficiency compared to control women. Similar results were found in the analysis of the baseline data.[43] Hence, the likelihood of a positive outcome is high in both women with thrombophilia and in controls. During prospective follow-up, 83 women with thrombophilia used heparin or oral anticoagulants during pregnancy. However, we could not draw solid conclusions on the benefit of thromboprophylactic treatment on the pregnancy outcome due to the differences in dose, type and starting point of treatment. As the risk of a first fetal loss without thromboprophylaxis was only mildly increased in this analysis, a strong beneficial effect of thromboprophylactic treatment is not likely.

Table 1 *Overview EPCOT data on the risk of a first and second venous thrombotic event*

	Retrospective data (Chapter I.1) Risk of a first event		Prospective data (Chapter I.2) Risk of a first event		Prospective data (Chapter I.3) Risk of a second event	
	Thrombophilia subjects	Controls	Thrombophilia subjects	Controls	Thrombophilia subjects with AC	Thrombophilia subjects without AC
Number of subjects	846	1212	575	1118	124	180
Number of subjects with event	139	15	26	7	7	44
Person years of all subjects	31660	51079	3194	6270	652	882
Incidence in %/year (95% CI)	0.4 (0.4-0.5)	0.0 (0.0-0.1)	0.8 (0.5-1.2)	0.1 (0.0-0.2)	1.1 (0.4-2.2)	5.0 (3.6-6.7)
Relative risk	16.4 (9.6-28.0)*		9.0 (3.8-21.1)**		0.2 (0.1-0.4)**	

Abbreviation: AC=anticoagulant therapy, CI=confidence interval *Adjusted for sex and center effects **Adjusted for sex, age at entry and center effects

Gene-gene interaction and gene mapping in a large protein C deficient kindred

Besides the EPCOT study, this thesis describes data from a large family with protein C deficiency. In this protein C deficient kindred, unexpectedly, a lack of interaction between the prothrombin G20210A mutation and the protein C 3363C mutation was found, and therefore no increase in the risk of venous thrombosis in family members who carried both mutations. We postulated a potential beneficial effect on activated protein C (APC) generation in carriers of the prothrombin G20210A mutation due to increased thrombin generation associated with this variant. This hypothesis could, however, not be substantiated as we found no association between the prothrombin G20210A variant and protein C activation, as measured indirectly by measuring plasma concentrations of APC in complex with protein C inhibitor (PCI) with a new and highly sensitive assay (Chapter I.5). For this analysis, blood samples were used from 195 family members, of whom 55 carried the protein C 3363C mutation, and 24 carried the prothrombin G20210A mutation. Protein C activity levels correlated positively with APC-PCI complex plasma concentrations (r=0.69). However, APC-PCI complex levels were not different for carriers of the prothrombin G20210A mutation compared with non-carriers. Therefore, our findings suggest that if the observed higher levels of thrombin generation and activity compensate for the impaired protein C pathway in this thrombophilic family, it must be by an alternative mechanism.

For the identification of genes or gene variants influencing the risk of complex diseases generally two main approaches are applied: linkage studies using data on related individuals (e.g., family studies) and association studies using data on unrelated individuals (e.g., case-control studies). Family studies are very useful for the identification of rare but relatively strong alleles, whereas case-control studies can be used to identify common (and generally also weaker) gene variants. In the protein C deficient kindred evidence was found for the presence of an unknown genetic defect, which strongly increased the risk of venous thrombosis in interaction with the protein C 3363C insertion in this kindred.[44] We started a search for unknown genes influencing thrombosis risk using the linkage study approach, when genetic screening of 34 candidate genes involved in hemostasis or inflammation provided no evidence that any of these candidate genes, including factor V Leiden and the prothrombin G20210A variant, was a good candidate for the unknown second gene promoting thrombophilia in this kindred.[45]

The identification of new genes influencing the risk of venous thrombosis can be performed by directly focusing on venous thrombosis or by focusing on intermediate phenotypes, i.e. plasma concentrations of known coagulation factors or markers of coagulation. Elevated plasma concentrations of several

procoagulant factors (e.g., factor VIII, factor IX and factor XI) have been shown to increase the risk of venous thrombosis[46-49], although no genetic variants underlying the elevated levels, besides ABO blood group for FVIII, have been discovered yet. The study of intermediate phenotypes, also known as quantitative traits, offers distinct advantages, as they are measured on a continuous scale and thus provide more information than a person's disease status, which is measured on a dichotomous scale (present/absent). In addition, quantitative traits probably are under more direct or exclusive genetic control than disease outcomes.

A first step before initiating a more detailed search for a genetic mechanism using linkage analysis is to determine the heritability of a certain trait (disease or quantitative trait). Chapter II.1 describes the results of an analysis performed to estimate the heritability of several hemostatic quantitative traits measured in blood samples from 330 members of the protein C deficient kindred. This analysis provided evidence for a genetic basis for the variation in levels of measures of thrombin activity (protein C activation peptide and fibrinogen peptide A), prothrombin, factor V, factor IX, protein C, protein Z, protein Z inhibitor, APC-PCI and APC in complex with α1-antitrypsin. The heritability estimates were largely similar to results found on the same coagulation factors in other studies.[50-53] Hemostatic factors showing a large genetic component are the best candidates to identify the genes influencing the variation in the plasma concentrations of these factors by linkage analysis. Chapter II.2 shows the evidence we found, using variance component linkage analysis, for two quantitative trait loci (QTLs) influencing the plasma concentrations of two markers of protein C activity: APC-PCI and APC-α1-antitrypsin complexes. For plasma concentrations of APC-α1-antitrypsin complex measured in 135 genotyped family members, we found evidence suggestive for linkage (defined as a lod score between 1.9 and 3.3) on chromosome 19 at 34 cM (lod score 3.3). In addition, we found suggestive linkage evidence for plasma concentrations of APC-PCI complex in 129 genotyped family members on chromosome 11 at 146 cM (lod score 2.6). However, as the linkage regions are too wide to be certain of linkage, we will finemap, i.e. narrow the regions on chromosomes 11 and 19, by adding microsatellite markers to the genome scan. In case candidate genes will be identified, the linkage analysis can be complemented with physical mapping for which single nucleotide polymorphisms (SNPs) in candidate genes are a first target. The search for variants will initially be focussed on coding regions (exons) and promotors of candidate genes as these are expected to influence risk of disease by variation in a quantitative trait. Candidate variants can subsequently be tested in association or family studies.

Genetic risk factors for venous thrombosis: key players or minor risk enhancers?

Summarizing the results of the prospective part of the EPCOT study we can conclude that the risk of first and recurrent venous thrombosis is increased in families with inherited thrombophilia, but that none of the genetic risk factors seems sufficient and necessary in heterozygous carriers for the development of venous thrombosis. However, single genetic minor risk enhancers with a low and incomplete penetrance can become key players in the presence of additional risk factors. This has been shown earlier in subjects with multiple genetic defects[16,54,55] and has been described by Vandenbroucke *et al.*, who showed a highly increased risk associated with factor V Leiden when women used oral contraceptives.[56] Therefore, the identification of new genetic risk factors, even those only minimally increasing the risk, can teach us more about different pathways involved in venous thrombosis and gene-gene and gene-environment interactions.

The search for new mutations should not be restricted to genes coding for hemostatic phenotypes associated with venous thrombosis. For example for factor VIII no genetic variations in clotting factor genes, such as of factor VIII or its carrier protein von Willebrand factor, have been found that are associated with plasma concentrations, whereas a large genetic basis for the variation in these levels was found.[57] In addition, in the LETS study evidence was found for a clustering pattern of pro- and anticoagulant factors by using principal-components analysis to explain relationships among correlated levels of coagulation factors.[58] This suggests the involvement of genes influencing the levels of several coagulation factors by modulating post-transcriptional or post-translational processes. As these pleiotropically acting genes will not be identified by the direct analysis of hemostatic candidate genes, linkage analysis seems to be the method of choice to identify these new genetic risk factors. However, despite the success of linkage analysis in identifying loci underlying diseases showing a Mendelian inheritance pattern (i.e. disorders caused by a single defective gene), linkage analysis is believed to be less powerful in finding genes with small effects underlying complex diseases.[59-61] As many genes are involved in the onset of a complex disease, family members who carry a single disease gene will not automatically develop disease, which precludes the identification of all carriers of the disease allele within a family: a prerequisite for efficiently performing linkage analysis.

We still are optimistic about applying linkage analysis to localize disease loci and QTLs in the protein C deficient kindred because only a few genes seem responsible for venous disease as most thrombotic episodes could be attributed to the co-occurrence of a second gene with protein C deficiency[44], and because of the high heritabilies found for quantitative traits in this kindred

(Chapter II.1). In addition, our study is thought to have a high relative efficiency (91%) for QTL mapping.[62] However, the main challenge will be replicating our linkage analysis results: disease alleles or alleles influencing quantitative traits might be only highly prevalent or penetrant in the protein C deficient kindred and might not show an association with disease or quantitative traits in population-based association studies. Genome-wide association studies might be more powerful to search randomly for genes, but at this point these studies are still too expensive and time-consuming, although this technological limitation could be overcome in the near future.[59-61] Until then, linkage analysis remains the only available analysis that allows scanning without an a priori hypothesis for new genes. The ultimate goal of extending our knowledge on genetic risk factors for venous thrombosis is not to apply gene-therapy, but to determine for which genetic risk factors patients should be screened, to inform patients on potential risk situations, and to provide optimal prophylactic treatment in those situations when assumed necessary. Manipulating the environment remains the most effective means to control complex common diseases.

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Samenvatting

Samenvatting

Veneuze trombose, oftewel ongewenste stolselvorming in de aderen die het bloed naar het hart vervoeren, is een frequente aandoening met jaarlijks één à twee nieuwe patiënten op de 1000 personen. Het komt meestal voor in de diepe aderen van het been, of in de longen na het loskomen van een stukje van het stolsel (longembolie). Er zijn vele factoren bekend die het risico op een veneuze trombose verhogen, zoals erfelijke afwijkingen in het stollingssysteem, hormonale veranderingen (bijv. bij zwangerschap of gebruik van de pil), weinig beweging (bijv. door dwarslaesie of gips), en orthopedische operaties. Van de patiënten die zich melden met een eerste veneuze trombose heeft ongeveer een kwart naaste familie met dezelfde aandoening. Erfelijke defecten die de kans op veneuze trombose verhogen zijn o.a. tekorten van de antistollingsfactoren proteïne C, proteïne S en antitrombine, en de factor V Leiden en protrombine G20210A mutatie. Zoals bij de meeste veel voorkomende ziekten is één risicofactor niet voldoende om de ziekte te veroorzaken. Zo is aangetoond dat veneuze trombose vaker voorkomt bij dragers van een erfelijk stollingsdefect met veneuze trombose in de familie dan bij dragers zonder veneuze trombose in de familie. In families met een stollingsdefect en een verhoogde aanleg voor veneuze trombose (trombofilie) wordt daarom de aanwezigheid van meer dan één genetisch defect verondersteld. Studies waarin het risico op veneuze trombose is onderzocht in families met trombofilie zijn schaars omdat familiaire defecten weinig voorkomen, met name in de natuurlijke antistollingsfactoren. Goede risicoschattingen zijn echter hard nodig om richtlijnen te kunnen formuleren voor het testen op deze defecten en het beslissen over het voorschrijven van preventieve antistollingstherapie in deze families. In 1994 is daarom een onderzoek begonnen om het risico op veneuze trombose te bepalen in individuen met erfelijke trombofilie: de "European Prospective Cohort study On Thrombophilia" (EPCOT). Onderzoekers uit verschillende Europese trombosecentra met expertise op het gebied van trombofilie deden mee aan dit onderzoek. In het eerste deel van dit proefschrift worden de resultaten beschreven van het EPCOT-onderzoek wat betreft het risico op een eerste of tweede trombose in deelnemers met een erfelijke tromboseneiging (Hoofdstuk I.1-3). Daarnaast hebben we binnen EPCOT gekeken naar het risico op miskramen bij vrouwen met een erfelijk stollingsdefect (Hoofdstuk I.4). Hoofdstuk II richt zich op het zoeken naar nieuwe nog onbekende genetische factoren die de kans op veneuze trombose verhogen. Slechts in 13% van de families met trombofilie zijn minstens twee defecten bekend, terwijl de aanwezigheid van meer dan één defect in alle families wordt verondersteld. De nu bekende defecten verklaren dus nog maar een klein deel van het risico op trombose in families met trombofilie.

Het risico op veneuze trombose en miskramen in het EPCOT onderzoek

In totaal werden 2838 deelnemers geïncludeerd in het EPCOT onderzoek, van wie 1626 met erfelijke trombofilie door de factor V Leiden mutatie of door deficiënties in proteïne C, proteïne S of antitrombine. De overige 1212 deelnemers waren vrienden of partners die werden onderzocht als vergelijkingsgroep (controlepersonen). Hoofdstuk I.1 beschrijft retrospectief de kans op trombose bij de deelnemers; vanaf de geboorte tot aan het begin van de studie. Van de 846 familieleden met erfelijke trombofilie die niet vanwege een trombose waren getest hadden 139 familieleden (16%) een trombose gehad voor inclusie in het onderzoek vergeleken met 15 van de 1212 controlepersonen (1%). Van de deelnemers met trombose hadden 58 familieleden (42%) en 2 controlepersonen (13%) al meer dan één episode doorgemaakt. De incidentie oftewel het jaarlijks aantal ziektegevallen voor inclusie in de studie was 4,4 per 1000 familieleden en 0,3 per 1000 controle personen. De incidentie van 0,3 per 1000 ligt dus iets lager dan de incidentie die werd gevonden in de literatuur voor de bevolking van één à twee nieuwe patiënten per 1000 mensen per jaar. De hoogste incidentie werd gevonden voor familieleden die meer dan één defect hadden (8,4 per 1000 per jaar). Voor familieleden met één bekend defect werd het hoogste risico gevonden bij familieleden met proteïne S deficiëntie (7,1 per 1000), en het laagste risico bij familieleden met de factor V Leiden mutatie (1,5 per 1000).
Na inclusie werden deelnemers gemiddeld 5,5 jaar prospectief gevolgd. Bij het begin van het onderzoek waren 575 familieleden en 1118 controlepersonen trombose-vrij en antistollingstherapie-vrij, van wie 26 familieleden (4,5%) en 7 controles (0,6%) een eerste trombose kregen in de vervolgjaren na de inclusie (Hoofdstuk I.2). Bij 15 familieleden (58%) en 3 controlepersonen (43%) ontstond de trombose in afwezigheid van bekende risicofactoren zoals operaties, zwangerschap of inactiviteit. De incidentie van een eerste trombose was 0,8% per jaar in familieleden en 0,1% per jaar in controle personen. Net als in hoofdstuk I.1 verschilden de incidenties per genetisch defect: de hoogste incidentie werd gevonden bij familieleden met antitrombine deficiëntie (1,7%/jaar) of meer dan één defect (1,6%/jaar), en de laagste incidentie bij dragers van factor V Leiden (0,1%/jaar). We vonden dus een hogere incidentie voor een eerste trombose in de analyse vanaf de inclusie (hoofdstuk I.2) dan in de analyse van gegevens over trombose voor de inclusie (hoofdstuk I.1). De incidenties uit hoofdstuk I.1 zijn echter waarschijnlijk lager, omdat de tijd tussen geboorte en inclusie in de studie werd genomen en het risico op trombose onder de 15 jaar te verwaarlozen is.
Hoofdstuk I.3 beschrijft de kans op een tweede trombose in de vervolgjaren na de start van de studie in familieleden met trombofilie en één veneuze

trombose voor het begin van de studie. Van de 180 patiënten die geen langdurige antistolling ontvingen, kregen 44 (24%) een tweede trombose vergeleken met 7 van de 124 (6%) patiënten die wel langdurige antistolling ontvingen. De incidentie zonder langdurige antistolling was 5,0% per jaar en dus veel hoger dan de incidentie van een eerste trombose (hoofdstuk I.2). De incidentie was echter niet hoger dan de incidentie in de literatuur voor patiënten met veneuze trombose zónder een trombosegeschiedenis in de familie. Net als in hoofdstuk I.2 werd de laagste incidentie gevonden in factor V Leiden dragers, en de hoogste incidentie bij individuen met antitrombine deficiëntie. De incidentie werd met 80% verlaagd door langdurige antistolling en was daarmee bijna gelijk aan de incidentie van een eerste trombose, maar het gebruik van langdurige antistollingstherapie resulteerde in een risico op ernstige bloedingen van 0.8% per jaar.
Uit de voorgaande hoofdstukken kan worden afgeleid dat er geen duidelijk voordeel bestaat in het voorschrijven van langdurige antistollingstherapie bij mensen met een erfelijk stollingsdefect. Ten eerste weegt het risico op een eerste trombose bij mensen met erfelijke trombofilie niet op tegen het risico op bloedingen tijdens het gebruik van antistolling zoals beschreven in de literatuur (1-3%). Ten tweede is het herhalingsrisico even groot bij mensen met of zonder een stollingsdefect en wordt voor de laatste groep geen langdurige antistollingstherapie voorgeschreven.
Naast het risico op trombose hebben we ook gekeken naar het risico op miskramen bij vrouwen met erfelijke trombofilie. In totaal werden 90 vrouwen die geen miskraam hadden gehad voor het begin van de studie zwanger in de jaren na de studie inclusie: 39 vrouwen met trombofilie en 51 controle vrouwen. Zeven van de 39 vrouwen met trombofilie (20%) en 7 van de 51 controle vrouwen (14%) kregen een miskraam. Er was geen verschil in de kans op een miskraam per type defect. De kans op een goede afloop van de zwangerschap was dus hoog voor beide groepen vrouwen.

Zoektocht naar nieuwe genen in een familie met proteïne C deficiëntie

Sinds 1985 wordt een grote familie (ongeveer 800 leden) met proteïne C deficiëntie gevolgd aan de universiteit van Vermont (USA). Ongeveer 25% van de familieleden heeft proteïne C deficiëntie door een bepaalde mutatie (3363C mutatie) in het proteïne C gen, en ongeveer 25% van deze dragers ontwikkelt een veneuze trombose. In 1998 werd bewijs gevonden voor de aanwezigheid van een onbekend gen dat samen met het proteïne C gen het ontstaan van veneuze trombose in de familie voor een groot deel verklaart. Er werden verschillende kandidaten getest, waaronder factor V Leiden en protrombine G20210A, maar zonder resultaat. Voor de protrombine G20210A mutatie was dit een onverwachte bevinding, aangezien de mutatie in deze familie vaker

voorkomt dan in de algemene bevolking (bij 13% in plaats van 2-4%) en deze mutatie vaak het risico op trombose verhoogt in combinatie met andere stollingsdefecten zoals factor V Leiden. De protrombine G20210A mutatie wordt in de literatuur geassocieerd met een verhoogde trombine aanmaak. Trombine activeert proteïne C, dus de protrombine G2010A mutatie zou via een verhoogde trombine aanmaak een gunstig effect kunnen hebben op de activering van proteïne C in familieleden met proteïne C deficiëntie. Door het complex van proteïne C met een remmer te meten als maat voor proteïne C activering testten we deze hypothese. We vonden echter geen verband tussen de protrombine G20210A variant en proteïne C activering in 195 familieleden, van wie 55 de proteïne C mutatie droegen en 44 de protrombine G20210A mutatie (Hoofdstuk I.5).

Voor de identificatie van nieuwe stollingsgerelateerde genen in deze proteïne C deficiënte familie richtten we ons op genen die de concentraties van stollingsfactoren en dus indirect de kans op veneuze trombose zouden kunnen beïnvloeden. Ten eerste hebben we bepaald of er wel een genetische basis is voor de variatie in de concentraties van verschillende stollingsfactoren in het bloed in 330 familieleden (Hoofdstuk II.1). We vonden bewijs voor een redelijk grote genetische basis voor o.a. concentraties van protrombine, factor V, factor IX, proteïne C en proteïne Z. Stollingsfactoren met een grote genetische component zijn de beste kandidaten voor het identificeren van genen die de concentraties in het bloed van deze stollingsfactoren beïnvloeden. Hoofdstuk II.2 toont de resultaten van een studie waarbij we genen hebben geprobeerd te koppelen aan de concentraties in het bloed van complexen die worden gemeten als maat voor proteïne C activiteit, namelijk geactiveerd proteïne C gebonden aan twee proteïne C remmers. Door in het genoom van elk individu op verschillende plaatsen een marker te bepalen, kan worden bepaald of de concentratie van een stollingsfactor gelijk wordt overgeërfd met een bepaalde marker waarvan de plaats bekend is. Met deze methode vonden we een aanwijzing voor een koppeling tussen een marker op chromosoom 19 en concentraties van het complex van geactiveerd proteïne C met de remmer α1-antitrypsine. Daarnaast vonden we een koppeling tussen een marker op chromosoom 11 en concentraties van het complex van geactiveerd proteïne C met "proteïne C remmer". Door het toevoegen van markers in het geïdentificeerde gebied kan vervolgens in meer detail worden nagegaan of er werkelijk een gen gekoppeld kan worden aan de gemeten concentraties.

Genetische risicofactoren voor veneuze trombose: schuldig of medeplichtig?

Uit de resultaten van hoofdstuk I.1 en I.2 kan worden geconcludeerd dat de kans op veneuze trombose verhoogd is in dragers van familiaire stollingsdefecten, maar dat de aanwezigheid van de meeste defecten niet onherroepelijk leidt tot het ontstaan van een veneuze trombose. De aanwezigheid van andere genetische risicofactoren of omgevingsfactoren kan het effect van deze stollingsdefecten op de kans op veneuze trombose hebben beïnvloed, zoals kan worden afgeleid uit het hoge risico op veneuze trombose dat werd gevonden in hoofdstuk I.2 en I.3 bij mensen die drager waren van verscheidene defecten. De identificatie van nieuwe genetische risicofactoren kan ons dus meer leren over hoe de interactie tussen verschillende risicofactoren uiteindelijk leidt tot het ontstaan van een trombose. Het uiteindelijke doel van het vinden van nieuwe genetische risicofactoren voor trombose is niet om gentherapie te kunnen toepassen, maar om informatie te krijgen over op welke genetische risicofactoren het best kan worden getest en om patiënten te kunnen informeren over eventuele risicosituaties en de mogelijkheden om in die situaties een trombose te voorkomen. Het beïnvloeden van omgevingsfactoren blijft nog altijd de meest effectieve methode om ziekte te voorkomen.

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Ar, zonder jou was dit boekje nooit geworden wat het nu is!

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 27 april 1976 te Gouda. Na het doorlopen van de middelbare school (Christelijk Lyceum Delft, VWO diploma 1994), begon zij aan de studie Biomedische Wetenschappen aan de Universiteit Leiden. Tijdens de studie heeft zij onder andere stage gelopen bij de afdeling Medische Farmacologie (Sylvius Laboratorium, Leiden) en de afdeling Klinische Epidemiologie (Leids Universitair Medisch Centrum). In 2000 zette zij haar stage-onderzoek bij de afdeling Klinische Epidemiologie voort als AIO onder leiding van Prof. dr F.R. Rosendaal. Tijdens haar AIO-schap heeft zij twee jaar gewerkt op de afdeling Pathologie aan de Universiteit van Vermont (Burlington, USA) onder leiding van Prof. E.G. Bovill. Tevens heeft zij in 2002 meegewerkt aan een door de Centers for Disease Control and Prevention (CDC, Atlanta, Georgia, USA) gefinancierd project over het nut van het testen op factor V Leiden en de protrombine G20210A mutatie bij patiënten met een eerste veneuze trombose, uitgevoerd door de Foundation for Blood Research (Portland, Maine, USA). Tevens heeft zij onder andere cursussen gevolgd van Prof. dr K.J. Rothman (Mediterranean School of Epidemiology and Statistical Methods in Biomedical Research, Crotone, Italië) en Prof. dr T.H. Beaty en Dr. K.Y. Liang (Johns Hopkins Bloomberg School of Public Health, Baltimore, USA). Sinds augustus 2004 werkt zij als post-doc op de afdeling Klinische Epidemiologie onder leiding van Prof. dr F.R. Rosendaal en Prof. E.G. Bovill.